Regulation of the proliferation and differentiation of human bone marrow stromal cells

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Regulation of the Proliferation and Differentiation of Human Bone Marrow Stromal Cells

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This thesis is dedicated to my Grandmother,

Molly Gibbons.

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Index

Summary

An improved understanding of the factors which regulate normal bone turnover is essential for effective management of many bone diseases, and for the treatment of bone defects and inherited disorders using autologous cell therapy. Involutional osteoporosis is characterised by a decrease in skeletal mass and structural integrity, and is thought to occur due to a reduction in the number of bone forming cells, osteoblasts, at sites of skeletal remodelling. Osteoblasts are derived from mesenchymal stem cells, termed colony forming units-fibroblastic (CFU-F), which are situated within the bone marrow stroma. In this study the influence of culture conditions and bone-anabolic agents on the colony formation, proliferation and differentiation of human bone marrow-derived CFU-F was determined *in vitro*, with particular emphasis on cells of the osteoblastic lineage.

Findings demonstrate that the continuous presence of L-ascorbate was essential for the proliferation of the clonogenic cells in culture. All factors tested had little influence on the number of colonies formed, and this was highly variable and dependent on the individual donor sample. Donor age provided little prediction of colony forming efficiency, although marrow stromal tissue from females was found to contain fewer osteogenic precursors than from males. Platelet-derived growth factor was demonstrated to be a candidate mitogen for the *ex-vivo* expansion of human bone marrow stromal cells that retain an immature phenotype but possess osteogenic potential, and might be used for tissue reconstruction and the healing of bone defects. In addition, further understanding of the osteoblast lineage is potentially attainable through conditionally immortalised cell lines derived from marrow stromal cells. Thus, this work has developed the understanding of factors that can be used to manipulate colony formation and expansion of human marrow stromal cells *in vitro*. This will be of importance in future investigation of bone physiology and pathology.

Abbreviations

Ab	antibody
ALCAM-1	activated leukocyte cell adhesion marker type 1
AP	alkaline phosphatase
ASC	L-ascorbic acid
ASP	L-ascorbic acid 2-phosphate
ATCC	American Type Culture Collection
β-GP	β–glycerophosphate
BLK	bone/liver/kidney
BMP	bone morphogenetic protein type 1
BMSC	bone marrow stromal cells
BMU	basic multicellular unit
BSA	bovine serum albumin
BSP	bone sialoprotein
cAMP	cyclic adenosine monophosphate
CBFA-1	core binding factor alpha-1
cDNA	complementary deoxyribonucleic acid
CFE	colony forming efficiency
CFU-F	colony forming unit-fibroblastic
CHO	Chinese hamster ovary cells
DAG	diacylglycerol
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagles medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphate
Dx	dexamethasone
ECACC	European Collection of cell cultures
ECL	enhanced chemiluminesence
ECM	extracellular matrix
EDTA	ethylenediamineotetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbant assay

ER	oestrogen receptor
FACS	fluorescent activated cell sorting
FCS	foetal calf serum
FDA	fluorescein diacetate
FGF-2	basic fibroblast growth factor
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde dehydrogenase
HBDC	human bone-derived cells
HBS	HEPES-buffered saline
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2- ethane sulphonic acid]
IC	isotype control
Ig	immunoglobulin
IGF-I	insulin-like growth factor type I
IGF-II	insulin-like growth factor type II
IGF-IR	IGF-I receptor
IGFBP	IGF-I binding proteins
IL	interleukin
IP_3	inositol triphosphate
LB	Luria-Bertani
mAb	monoclonal antibody
MB	methylene blue
MEM	Eagle's minimum essential medium
MFI	mean fluorescent index
mRNA	messenger ribonucleic acid
MT	metallothionein
n	number
NBF	neutral buffered formalin
NC	nitrocellulose
NSAIDS	non-steroidal antiinflammatory drugs
OC	osteocalcin
OD	optical density
OSF-2	osteoblast specific factor type 2
p-NP	para-nitrophenol
p-NPP	para-nitrophenyl phosphate
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PGE_2	prostaglandin E_2
PGHS	prostaglandin G/H synthase
PI	propidium iodide
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PPARγ	peroxisome proliferator-activated receptor gamma
PTH	parathyroid hormone
PTHR	parathyroid hormone receptor
R-PE	R-phycoerythrin
RBC	red blood cells
RGD	arginine-glycine-aspartic acid
RT	reverse transcriptase
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SE	standard error
SRCC	Spearmans rank correlation coefficient
SSC	sodium chloride, sodium citrate buffer
SV40	simian virus 40
TAE	tris-acetate, EDTA buffer
TE	tris-EDTA buffer
TGFβ	transforming growth factor beta
UV	ultra-violet
VCAM-1	vascular cell adhesion molecule type 1
WM	whole marrow

Chapter 1

Introduction

1.1 Bone

Bone is a highly specialised connective tissue. Functionally, bone serves three main roles in vertebrates: it acts as a mechanical support for the body and a site of muscle attachment for locomotion, it provides a protective barrier safeguarding vital organs such as the brain, lungs and spinal cord from external trauma, and it is a metabolic organ, providing a reservoir for the body calcium and phosphorous. Additionally, bone marrow serves as a site of haematopoiesis and a source of connective tissue stem cells (Baron, 1993).

Under non-pathological conditions the adult skeleton exists as two architecturally divergent bone types. Cortical bone forms the external surface of all bones and represents 80% of total bone mass and has a porosity of ~10%. It primarily fulfils the mechanical and protective function of the marrow. Trabecular bone has a porosity of ~75%. It occurs in the metaphyses and epiphyses of long and flat bones and forms the bulk of the vertebral bone mass. It is composed of a network of anatomising struts that are continuous with the inner surface of the cortex (endosteum) and are orientated to provide maximum resistance to tensile and compressive forces. The spaces enclosed by these thin trabeculae are filled with bone marrow (Baron, 1993).

Like other sub-types of connective tissue, bone is composed of cells embedded in a matrix of osteoid and fibres. The cells and matrix of bone tissue are organised to maximise the relationship between structure and function. Bone rigidity is provided by the deposition of inorganic salts such as calcium phosphate and calcium carbonate in the osteoid. In the matrix these form hydroxyapatite crystals which may be ionised when required to provide calcium and phosphate to satisfy transient bodily needs. The organisation of collagenous fibres in the matrix contribute towards bone strength and flexibility.

The bones of the skeleton have two embryological origins. Flat bones, particularly those of the skull and face, are formed by intramembranous ossification, following the direct differentiation of mesenchymal cells into osteoblasts. In the bones of the axial and apendicular skeleton, cartilaginous tissue formed by chondroblasts derived from the condensed embryonic mesenchyme, is progressively replaced with mineralised tissue by a process termed endochondral ossification. A more complete description of these processes is not appropriate in the context of this thesis and the interested reader is referred to a review chapter of Marks and Hermey (1996).

1.2 Bone remodelling in the adult skeleton

The structure and functional integrity of bone in the adult skeleton is maintained by a continuous, life-long process termed remodelling. This process serves to prevent the accumulation of microdamage and allows the skeleton to adapt to changes in mechanical loading (Pead and Lanyon, 1989). This is achieved through a delicate balance of bone resorption and formation performed respectively by osteoclasts and osteoblasts. These two closely coupled cellular activities are highly co-ordinated in space and time, and are responsible for renewing the skeleton while maintaining its anatomical structure. The role of the skeleton as a mineral reservoir is also facilitated by the remodelling process (Manolagas and Jilka, 1995).

Osteoclasts, osteoblasts and their respective progenitor cells active in a remodelling cycle comprise the basic multicellular unit (BMU). The size, shape and individual identity of a BMU during its life-span, is co-ordinated spatially and temporally, and the relationship between its components maintained by the continued sequential recruitment of new bone cells (Puzas, 1993). The demand for osteoblasts is created by the number, size and location of the cavities made by osteoclasts. Under normal conditions remodelling takes place as a series of events summarised in figure 1.1. The duration of each of these events is indicated in figure 1.2.

1.2.1 Activation

In response to damage and/or changes in mechanical loading, bone lining cells retract, exposing the bone surface and allowing access to osteoclasts. Surface protein is removed by the release of a variety of proteolytic enzymes (Cheng *et al,* 1991, Chow and Chambers, 1992). Soluble mediators and cellular stimuli then recruit osteoclast precursors to the exposed bone surface (Fuller *et al*, 1992).

1.2.2 Resorption

As the mononuclear precursors approach the bone surface they fuse and become multinucleated osteoclasts. These become polarised and develop membrane specialisations (sealing zone and ruffled border) adjacent to the bone surface. Protons and acid proteases are secreted across the ruffled border into the extracellular space bonded by the sealing zone. Release of hydrogen ions and lysosomal enzymes by the activated osteoclasts degrades the mineralised matrix. During the resorption process previously sequestered growth and chemotactic factors are released in an active form and are thought to mediate recruitment and proliferation of osteoblast precursors. During the period of reversal, osteoclast activity is halted by mechanisms that remain poorly understood but which are thought to include an increase in intracellular calcium (Zaidi *et al*, 1992) and the release of TGFβ or related peptides from the bone matrix (Chenu *et al*, 1988, Pfeilschifter *et al*, 1990a).

1.2.3 Reversal

The reversal phase is characterised by the disappearance of osteoclasts and the appearance of a poorly characterised population of mononuclear phagocytes (Raisz, 1993). These appear to function in the removal of residual matrix protein, the deposition of the cement line, and possibly the coupling of resorption to formation.

1.2.4 Formation

This phase is characterised by the differentiation of osteoblasts which then proceed to deposit an amount of bone equivalent to that removed by the osteoclasts. In addition to producing the unmineralised bone (osteoid) osteoblasts are responsible for initiating its mineralisation by a mechanism that remains poorly understood.

It is important to appreciate that remodelling is the predominant form of bone turnover in the adult skeleton and that all losses or gains in bone mass are therefore a consequence of a focal imbalance between activities of osteoclasts and osteoblasts during each remodelling cycle.

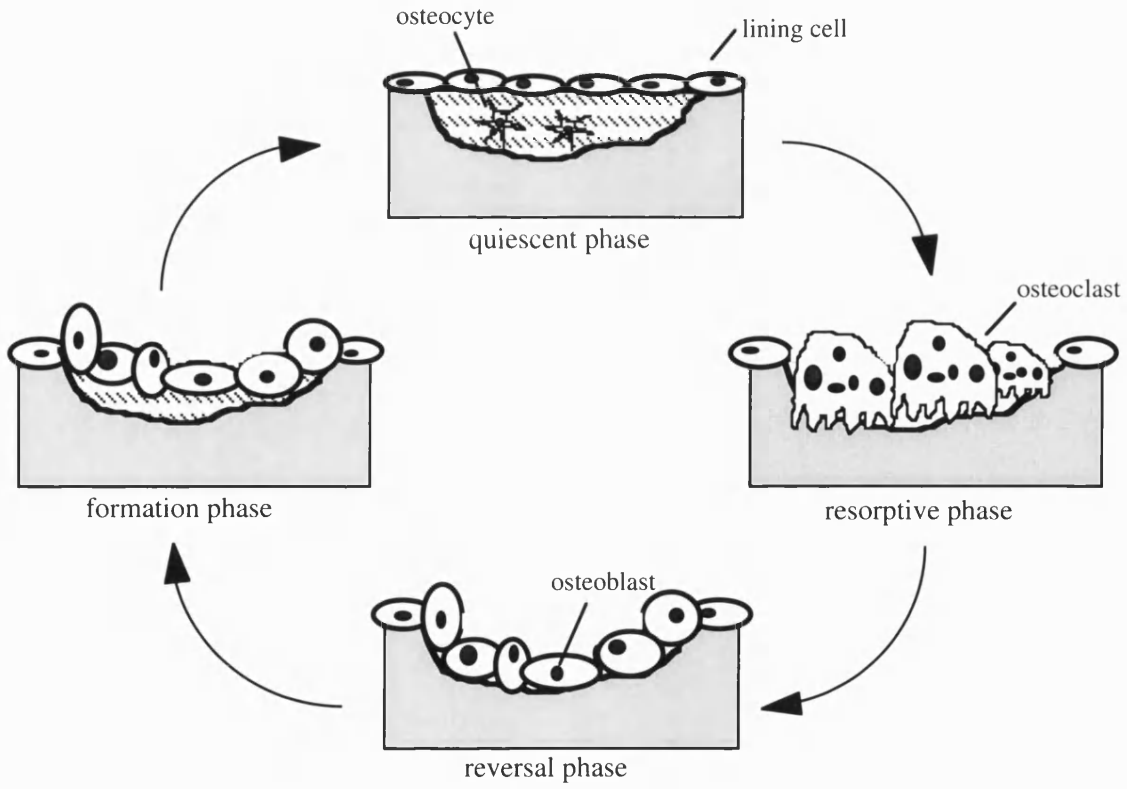


Figure 1.1 Illustration of the bone remodelling cycle

Adapted from Baron, 1990.

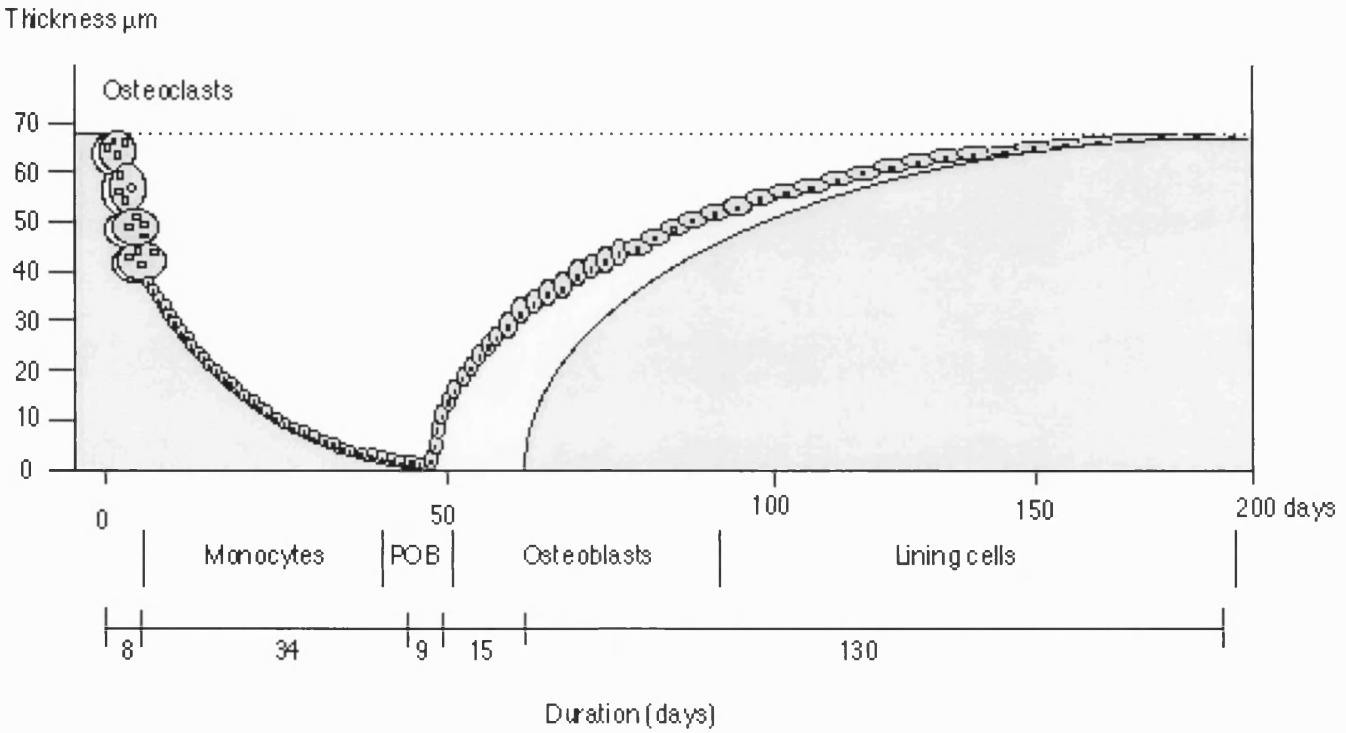


Figure 1.2 Illustration of the events in the basic multicellular unit

Illustration of the events in the basic multicellular unit with the duration of each event stated in days (adapted from Remagen, 1989). POB, preosteoblasts.

1.3 Osteoporosis

A disease that occurs due to disruption of bone turnover is osteoporosis. Osteoporosis, or brittle bone disease, is the most common metabolic disorder in developed countries. The prevalence of osteoporosis increases with age (Baron, 1993), and affects 1 in 4 post-menopausal women and 1 in 8 elderly men. Osteoporotic patients develop an increased risk of fracture, particularly of the wrist, spine and hip, and the disease is implicated in more than 1.5 million fractures a year. Consequently, osteoporosis is a serious public health problem, particularly in the rapidly growing population of individuals over the age of 65. In 1994 it was calculated that the cost of treating osteoporotic fractures in the UK was 750 million pounds per annum (National Osteoporosis Society, 1996).

The World Health Organisation has defined osteoporosis as a decrease in bone mineral density of 2.5 standard deviations below the mean value for young adult females, with an accompanying deterioration of the skeletal microarchitecture. Two broad categories are recognised, primary and secondary, depending on the underlying aetiology. Primary osteoporosis is further categorised post-menopausal osteoporosis, type I, and age-related osteoporosis, type II (summarised in table 1.3), and is dependent on several factors including the age of onset, sex, level of oestrogens, and cellular mechanisms of bone loss.

	Primary Osteoporosis			
	Affects	***Cause***	***Bone loss***	***Fractures***
Type I	Post-menopausal women	Oestrogen deficiency	Mainly trabecular	Vertebral + Radial
Type II	Men and women	Ageing	Cortical + trabecular	Hip

Table 1.3 Summary of the subtypes of primary osteoporosis

In normal subjects, peak bone mass is achieved during the fourth decade, and remains relatively constant in both genders until middle age. At menopause women undergo an accelerated transient phase of bone loss that is most apparent over the subsequent decade and accounts for cancellous bone losses of 20-30% and cortical bone losses of 5-10% (Riggs and Melton, 1986). The cellular hallmark of the osteopenia caused by oestrogen deficit is an increase in bone turnover, with resorption increased to a greater extent than bone formation (Balena *et al*, 1993, Han *et al*, 1997). The accelerated phase of bone loss diminishes exponentially with time.

Type II osteoporosis is manifested as a slow rate of bone loss due to changes in cellular response and function with ageing. Over a lifetime this accounts for losses of about 20-30% of cancellous bone and about 5-10% of cortical bone in both genders and continues indefinitely (Riggs and Melton, 1986). There is increasing evidence that the reduction in bone mass occurring in the osteoporotic skeleton does not result purely from an increase in bone resorption. Osteopenias associated with ageing appear to be due to a decrease in osteoblastic cell formation and defective recruitment of osteoblasts to the remodelling site. Factors implicated as causes of bone loss in the ageing population include secondary hyperparathyroidism, changes in local cytokines or systemic growth factors, and nutritional deficiency of vitamin D (Riggs *et al*, 1998).

Some of the causes of secondary osteoporosis are shown in table 1.4. Of these perhaps the factor of most importance in this study is that of glucocorticoid excess. This can arise due to endogenous hypersecretion (Cushings disease) or prolonged administration of high doses of exogenous glucocorticoids, for example in the treatment of chronic inflammatory disorders.

Some risk factors for secondary osteoporosis	
Genetic	White or Asian decent Small, thin or tall, lean build Family history Females more susceptible than males
Endocrine	Early menopause Oestrogen deficiency Not giving birth
Nutritional	Calcium and vitamin D intake deficiency Excessive alcohol consumption
Drugs	Glucocorticoids, thyroid medications, anticonvulsants, anticoagulants
Lifestyle	Cigarette smoking Physical inactivity or immobilisation

Table 1.4 Risk factors for secondary osteoporosis

There is now general agreement that the rapid phase of bone loss associated with the loss of ovarian function in women is due to an increase in the number, activity and functional lifespan of osteoclasts (Sarma *et al,* 1998). This pattern of bone loss does not persist, however, and during the later stages of the disease and in age-related osteoporosis the predominant mechanism of bone loss appears to be a reduction in bone formation due primarily to a reduction in the number of osteoblasts. This is also believed to be the predominant mechanism of bone loss that is a frequent complication of chronic rheumatoid arthritis (Compston *et al*, 1989, Compston and Croucher, 1991). It follows therefore, that future prospects for the prevention and treatment of osteoporosis will depend on a precise understanding of the origin of osteoblasts in the adult skeleton and the identification of the factors that regulate their recruitment, proliferation and differentiation.

1.4 The cells of bone

1.4.1 Osteoclasts

The cell type responsible for bone resorption is termed the osteoclast. These are giant multinucleated cells which secrete protons, and acidic and neutral proteases including collagenase, across their ruffled border into an extracellular hemivacuole adjacent to the bone surface. In the acidic microenvironment thus formed, the bone is demineralised, rendering the organic phase susceptible to proteolytic attack. The products of resorption are endocytosed at the ruffled border, transcytosed across the cell, and secreted from its basolateral surface (Nesbitt and Horton, 1977). The ultimate fate of osteoclasts is death by a process that bears the hallmarks of apoptosis (Hughes and Boyce, 1997). The ruffled border also provides a large surface area for the absorption of the degraded bone matrix. Osteoclasts develop via the fusion of circulating mononuclear precursors derived from haematopoietic stem cells (Kerby *et al*, 1992, Baron, 1993). It is currently unclear, however, whether the osteoclast is the product of a distinct cell lineage or merely an 'offshoot' of the monocyte/macrophage lineage (Suda *et al,* 1996). The development of osteoclastic precursors in the marrow is regulated by growth factors and cytokines that are produced by cells of the stromal/osteoblastic lineage (Manolagas and Jilka, 1995).

1.4.2 Osteoblasts

Osteoblasts are primarily responsible for the synthesis, deposition, and organisation of the bone extracellular matrix. Fully-differentiated osteoblasts are found adjacent to bone forming surfaces lining a layer of as yet unmineralised bone matrix (osteoid). They are cuboidal in shape and possess an eccentric nucleus with prominent nucleoli, a well developed golgi apparatus, and an abundance of ribosomes both free and associated with endoplasmic reticulum. At the end of their functional lifespan the majority of osteoblasts disappear, presumably by the process of apoptosis (Zimmerman, 1992, Jilka *et al*, 1998).

1.4.3 Osteocytes

As bone tissue is formed a proportion of the osteoblast population becomes entombed within the extra-cellular matrix. These entombed cells are termed osteocytes, and are the most abundant cell type in bone tissue (25,000/mm^3). They possess long cell processes that extend through canaliculae in the bone matrix and make contact with other osteocytes and cells on bone surfaces. Evidence suggests that osteocytes are the mechanosensory cells of bone and play a central role in the dynamic adaptation of the tissue (van der Plas *et al*, 1994).

1.4.4 Bone lining cells

Terminally-differentiated osteoblasts may also become lining cells. These are present on quiescent endosteal bone surfaces, and although their function is unknown, they may be involved in osteoclast recruitment and/or regulate their access to the bone surface. The results of recent studies suggest that these cells may also have the ability to become biosynthetically active again, providing a source of osteoblasts at times of crises such as during fracture repair (Dobnig and Turner, 1995, Leaffer *et al*, 1995).

1.5 Factors affecting bone turnover

Bone turnover is regulated by an elaborate interplay between systemic hormones and a variety of locally-produced growth factors and cytokines, many of which become sequestered in an inactive form in the bone matrix (Rodan, 1991, Whitfield and Morely, 1995). Many of these factors may act as couplers between bone resorption and formation. Other factors influence the proliferation and differentiation of the bone cells. The local and systemic factors that regulate bone turnover include calcium regulating hormones, systemic hormones, cytokines, prostaglandins and growth factors. An in-depth description of all of the factors, systemic and local, that participate in this process is beyond the scope of this thesis. Instead, specific emphasis will be placed upon those factors employed in this investigation and for a wider overview the reader is referred to Bilezikian *et al,* 1996.

1.5.1 Glucocorticoids

Glucocorticoids have marked *in vivo* effects on bone and mineral metabolism, and osteoporosis is a common complication of patients with glucocorticoid excess, which has been attributed to increased bone resorption and decreased bone formation (Delany *et al*, 1994). The effect on bone resorption is indirect and is perhaps related to secondary hyperparathyroidism (Bikle *et al*, 1993). The decrease in bone formation is thought to occur through a direct inhibition of osteoblastic function *in vivo* (Reid *et al*, 1986). Paradoxically however, at physiological concentrations *in vitro,* glucocorticoids have been shown to promote osteogenic differentiation in primary marrow stromal cultures from a number of species and to induce formation of mineralised nodules in rat marrow stromal cell cultures

(Maniatopoulos *et al*, 1988, Aubin *et al,* 1990, Bellows *et al*, 1990, Leboy *et al*, 1991, Davis *et al*, 1993, Beresford *et al,* 1994, Croisille *et al*, 1994, Cheng *et al*, 1994, Locklin *et al*, 1995). A decreased pharmacological dose *in vivo* can also stimulate bone formation, although this effect is species dependent (Chen *et al*, 1983, Beresford *et al*, 1994, Lian *et al*, 1997, Bellows *et al*, 1998). The effects of glucocorticoids are described in chapter 3.

1.5.2 Prostaglandin E_2

Bone cells, particularly osteoblasts, are abundant producers of prostaglandins, and prostaglandins of the E series were first found to be potent bone resorbers (Raisz *et al*, 1990). The increase in bone resorption that occurs with immobilisation and inflammation can be reduced by non-steroidal antiinflammatories (NSAIDS) which inhibit bifunctional prostaglandin G/H synthase (PGHS, Pilbeam *et al*, 1995). Prostaglandins have since been shown to have both biphasic stimulatory and inhibitory effects on bone formation in cell and organ culture (Raisz and Fall, 1990, Mori *et al*, 1992). The effects of PGE_2 on bone formation are described in further detail in chapter 4.

1.5.3 Growth factors

The bone mineral matrix is the most abundant source of growth factors in the body. During osteoclastic resorption, sequestered growth factors are released from the matrix into the extracellular fluid in an active form where they are available to act on the local cell populations or enter the circulation. The growth factors are produced by many of the cell types present in bone, including osteoblasts, endothelial cells and macrophages. Osteoblasts also express receptors for the majority of these growth factors, indicating that they can function in an autocrine as well as paracrine manner. A large number of growth factors have been identified in skeletal tissue and the fact that there are so many makes it difficult to assess their individual roles. Many of the growth factors and cytokines exert influences on the production of other factors, and/or their receptors, with which they can interact positively or negatively. Thus the effects of the growth factors on bone turnover are highly complex and it has often proved difficult to distinguish between their direct and indirect actions. A further complexity is that their production and/or incorporation into the bone matrix is affected by ageing and disease status, frequently resulting in adverse changes in resorption and formation. Growth factors associated with the control of osteoblast activity are summarised in table 1.5, and those that have been used in this thesis are discussed below.

Growth factor	kDa	Concentration in matrix (ng/g)	Proliferation*	Differentiation*
Insulin-like growth factor-II	7.5	1500	I	I
Transforming growth factor β	50	450	I	I
Insulin-like growth factor-I	7.7	100	I	I
Platelet-derived growth factor	35	60	I	D
Fibroblast growth factor	33	60	I	D

Table 1.5 Summary of the effect of growth factors on osteoblasts

Adapted from Canalis *et al*, 1989, Baylink *et al*, 1993. * I, increase, D, decrease.

1.5.3.1 Insulin-like growth factor type I

IGF-I and II are abundant, anabolic peptides, which are structurally and functionally related to insulin (Delany *et al*, 1994). IGFs are available to skeletal tissues through *de novo* synthesis by bone cells and by the release of stored peptide from the bone matrix. Circulating IGF-I and IGF-II constitute a family of growth hormone dependent insulin-like peptides that are bound to specific IGF binding proteins (IGFBP) and mediate the growth promoting actions of growth hormones. Bone cells produce IGF-I, IGF-II and all known IGFBP, except IGFBP-1, which can positively and negatively influence IGF actions (Mohan, 1993). IGF-I is expressed by mature osteoblasts with little or no expression in preosteoblasts or osteocytes (Middleton *et al,* 1995), and its production is subject to regulation by systemic hormones including parathyroid hormone (PTH). *In vitro* IGF-I stimulates osteoblast-like cell proliferation and protein synthesis as well as the recruitment and differentiation of osteoblast (Shinar *et al*, 1993, Middleton *et al*, 1995, Wang *et al*, 1995) and osteoclast (Mochizuki *et al*, 1992) precursors. IGF-I receptors have also been found on osteoclasts actively engaged in bone resorption (Middleton *et al,* 1995). Pharmacologically administered IGF-I has been shown to increase the level of bone formation markers in the circulation (Ebeling *et al*, 1992).

1.5.3.2 Platelet-derived growth factor

A growth factor with significant growth potential in bone is platelet-derived growth factor (PDGF). The principal polypeptide in mammalian serum, it is known to have growth promoting activity for a variety of target cells of mesenchymal origin (Ross *et al,* 1974, Heldin *et al*, 1992). The matrix bound PDGF fraction, released during bone resorption, has been postulated to promote the migration and/or proliferation of osteogenic precursors associated with the marrow the stroma (Centrella *et al*, 1989, Pfeilschifter *et al,* 1990, Gilardetti *et al*, 1991). PDGF induces the formation of new bone *in vivo* (Lynch, 1989). The activity of PDGF released *in vivo* may be modulated by association with a high molecular weight protein plasma binding component thought to be α2-macroglobulin (Huang *et al,* 1984, Raines *et al,* 1992, Cochran *et al*, 1993). PDGF is known to cause its effects through interaction with other mesenchymal growth factors such as IGF-I. Considered as a whole, current knowledge suggests that is unlikely that PDGF A or B play a role in the maintenance of bone mass, however, the actions of PDGF on bone cell replication suggest a possible role in fracture healing and repair.

1.6 Osteogenic precursors and the stromal system of bone and marrow

In the postnatal vertebrate, osteogenic precursors are found associated with the bone marrow stroma. This is composed of a heterogeneous mixture of haematopoietic and stromal cells surrounded by a delicate supporting mesh of collagen fibres and other extracellular matrix components (Bianco and Riminucci, 1998). The whole tissue is richly supplied with thin-walled blood vessels. In this environment the precursor cells are in intimate contact with the endocortical and cancellous bone surfaces as well as with marrow monocytes, haematoprogenitors and the endothelial cells lining the blood vessels of the extra vascular intersinusoidal space (Jilka, 1998).

Bone marrow stromal cells (BMSC) are essential for the formation of the bone and the haematopoietic microenvironment, and direct influences between stromal cells and haematopoietic cell growth have been described (Song *et al*, 1985, Dexter, 1982). It is known that the two populations interact, with each other, and with the extracellular matrix, through a variety of adhesion molecules. Although bone precursor cells coexist among a population of haematopoietic progenitor cells, it is currently accepted that they are ontogenically distinct, and do not, as has frequently been suggested, share a common origin in the postnatal vertebrate (Simmons *et al*, 1987, Beresford, 1989).

1.6.1 Colony Forming Units-Fibroblastic (CFU-F)

The pioneering studies of Friedenstein *et al* (1970) demonstrated that osteogenic precursors are derived from a population of cells termed Colony Forming Units-Fibroblastic (CFU-F), that exist in close association with the soft, fibrous tissue of the marrow stroma and are concentrated near the bone surfaces (Beresford, 1989). These were first identified on the basis of their ability to form fibroblastic colonies in cultures of marrow cells (Friedenstein *et al*, 1970, each of which is derived from a single cell type (Friedenstein *et al*, 1976, Friedenstein, 1980). Friedenstein later showed (1987) that marrow-derived CFU-F reproducibly form an osteogenic tissue comprised of cartilage and bone when transplanted *in vivo* within diffusion chambers. Subsequently, marrow stromal cells from a number of species have since been shown to form bone in assays of osteogenesis (Benayahu *et al*, 1989, Mardon *et al*, 1987, Nakahara *et al*, 1990, Gundle *et al*, 1995).

A subset of the CFU-F population exhibit stem cell-like characteristics in that they are capable of extensive self-renewal, and of giving rise to cells of multiple marrow stromal cell lineages including those necessary for the formation of bone and the recreation of the haematopoietic microenvironment (Dexter *et al*, 1977, Patt *et al*, 1982). More recently, Kuznetsov *et al* (1997) have shown that even after long term cultivation and multiple passages *in vitro,* human marrow stromal cells form at least five types of connective tissue upon transplantation *in vivo.*

The results of several studies indicate that cells of the different marrow lineages show considerable plasticity of phenotype (Bianco and Riminucci, 1998). Perhaps the best example of this is the interconversion of marrow adipocytes into bone forming osteoblasts (Beresford *et al*, 1992). Considerable progress has been made in recent years concerning the transcription regulation of lineage commitment. Thus MyoD (Filvarov and Derynck, 1996), peroxisome proliferator-activated receptor gamma (PPARγ, Tontonoz *et al,* 1994, Nuttall *et al*, 1998) and core binding factor alpha-1 (CBFA-1, Banerjee *et al*, 1997) have been identified as key regulators of the commitment of myoblasts, adipocytes and osteoblasts respectively (Rodan and Harada, 1997).

In vitro investigations have showed that CFU-F are a heterogeneous population of stem and progenitor cells which vary both in their potential to differentiate into multiple phenotypes, and in their sensitivity to inductive factors (Friedenstein *et al*, 1976, Owen, 1988, Kuznetsov *et al,* 1997a). Commitment and differentiation of the progenitors can be modified at the colony level, and under the appropriate culture conditions, colonies of specific lineages may be generated. However, the factors which regulate lineage commitment and by inference, therefore, the expression of the genes for these transcription factors, have yet to be fully elucidated in detail.

1.6.2 The differentiation of cells of the osteoblast lineage

Upon receipt of an appropriate signal, it is thought that the stem cells from which osteoblasts ultimately derive give rise to proliferative progenitors for the specific cell lineages, sometimes referred to as transit amplifying cells (Hall and Watt, 1989). The progenitor cells then undergo a number of population doublings to increase the size of the precursor population prior to cellular maturation. The existence of a progenitor cell compartment amplifies the effect of each stem cell division so that a relatively small number of rounds of stem cell division results in the production of a large number of terminally differentiated cells. A diagrammatic representation of the differentiation of cells of the osteoblast lineage is shown in figure 1.6. Following commitment, it has been calculated that a osteoprogenitor cells goes through 6-8 population doublings (Bellows and Aubin, 1989) before exiting the cell cycle and acquiring the functional characteristics of the mature osteoblast.

1.7 Stage specific markers of osteoblast differentiation

The differentiation of osteoblasts is a multistep process proceeding through defined stages (Aubin *et al*, 1993). Studies *in vitro* and *in vivo* established that the expression of bone related proteins, cell surface antigens and the receptors for systemic hormones and local factors, is ordered and sequential during the course of osteoblast maturation (reviewed in Stein *et al,* 1990, Owen *et al,* 1990). The earliest identifiable precursors produce low levels of collagens type I and III. The upregulation of collagen type I production and concomitant down regulation of collagen III is essential for proliferation and further differentiation. Once differentiation has begun, the other osteoblast marker genes are activated in a clear temporal sequence (Stein *et al*, 1990). Cells which are committed to the osteoblast lineage express alkaline phosphatase during the proliferative stage. Post-proliferatively there is an up-regulation in the expression of genes that support the maturation and organisation of the bone extracellular matrix (e.g. osteonectin). Subsequently genes that facilitate extracellular mineralisation (osteopontin and osteocalcin) are expressed. The mature osteoblast phenotype is characterised by high levels of expression of type I collagen, alkaline phosphatase, osteopontin, osteonectin, bone sialoprotein and osteocalcin, and most notably the ability to direct hydroxyapatite mineralisation of its extracellular matrix.

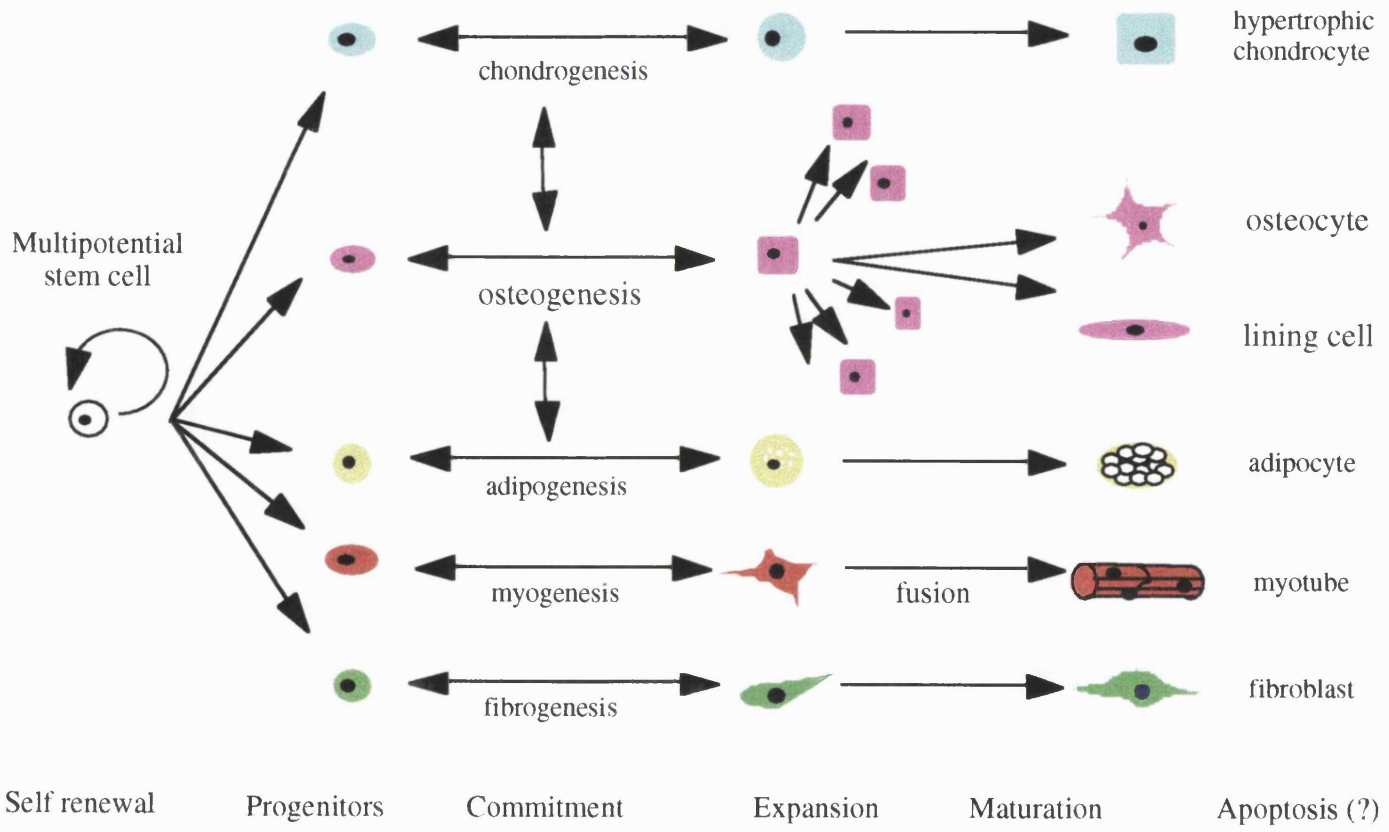


Figure 1.6 The differentiation pathways of mesenchymal stem cells

It has been shown in culture that expression patterns of bone-related proteins are also regulated in a temporal manner during the successive developmental stages (Choi *et al*, 1996). These stages can be followed from CFU-F through to mature osteoblasts via detection of specific marker proteins and for their messenger RNAs (figure 1.7). The definitive test for the differentiation of osteoblasts remains, however, the formation of a well ordered extracellular matrix that mineralises (Beresford *et al*, 1993). A brief description of the most commonly used stage specific markers of osteoblast differentiation follows.

1.7.1 Core binding factor A-1 (CBFA-1)

One potential marker of osteoblastic commitment and differentiation is the recently discovered osteoblast specific factor type 2 (OSF-2) otherwise known as the human core binding factor alpha-1 (CBFA-1). CBFA-1 is one of a three member family of mammalian transcription factors which is related to the drosophyla segmentation gene product, runt (Banerjee *et al*, 1997). In recent studies this protein has been shown to bind an osteoblast specific cis-acting element termed OSE-2 in the osteocalcin promoter (Geoffroy *et al*, 1995, Banerjee *et al*, 1995), and is an example of an osteoblast specific transcription factor. CBFA-1 expression is initiated in the mesenchymal condensation of the developing skeleton, is strictly restricted to cells of the osteoblast lineage thereafter, and is regulated by osteoblastic differentiating agents such as bone morphogenetic proteins (Ducy *et al*, 1997). Heterozygous loss of CBFA-1 function in humans occurs in the skeletal disorder cleidocranial dysplasia (CCD, Otto *et al*, 1997, Rodan and Harada, 1997). This is an autosomal-dominant condition characterised by hypoplasia/aplasia of clavicles, patent fontanels, short stature and supernumerary teeth. Homozygous inactivation of the cbfa-1 gene has revealed its critical role in bone formation and knockout mice have a complete lack of ossification due to the maturational arrest of osteoblasts (Komori *et al*, 1997).

1.7.2 Stromal cell marker, STRO-1

The monoclonal antibody STRO-1 was generated by Simmons and Torok-Storb (1991) and recognises an antigenic epitope present on a subset of assayable CFU-F. STRO-1^+ CFU-F proliferate extensively and give rise to cells of multiple marrow stromal cell lineages, including osteoblasts (Simmons and Torok-Storb, 1991, Gronthos *et al*, 1994). STRO-1 antibody recognises a trypsin-resistant, cell surface antigen of unknown identity. The results of recent studies suggest that the antigen is expressed during the early stages of osteoblast differentiation up to and including the stage at which alkaline phosphatase is first expressed (Stewart *et al*, 1998). As the cells undergo further maturation, however, its expression is down-regulated.

1.7.3 Alkaline phosphatase

Alkaline phosphatase (AP) EC 3.1.3.1, is a ubiquitously expressed ectoenzyme, the physiological function of which is essentially unknown. In the human, three isoenzymes exist. These are the intestinal, placental and bone/liver/kidney (BLK) forms which are encoded by distinct genes. The BLK enzymes differ only by post-translation modification of cell surface carbohydrate moieties, and the bone isoform can be identified experimentally by lectin binding properties (Harris, 1990). The enzyme is covalently bound to phosphatidyl inositol complexes in the plasma membrane where it functions to hydrolyse monophosphate esters at alkaline pH (Aubin *et al*, 1993). In bone, AP is thought to be important in the process of skeletal mineralisation, confirmed by the finding that a mutation in its gene is the cause of hypophosphatasia, a disease characterised by defective skeletal mineralisation (Cheng *et al,* 1996). During bone formation a proportion of AP is released into the circulation and, clinically, serum alkaline phosphatase is the most commonly used marker of bone formation. AP is widely accepted as a marker of osteogenic differentiation which increases in activity as the cell population shifts to a more differentiated state. As such, it is routinely used in *in vitro* experiments as a marker of osteoblast differentiation (Aronow *et al*, 1990). An increase in AP expression is one of the earliest indications that cells have an initiated programme of osteogenic differentiation and expression persists through cell stages up to and including osteoblasts, but is down-regulated in lining cells and osteocytes.

1.7.4 Parathyroid hormone/Parathyroid hormone-related protein receptor

Parathyroid hormone binds to specific, G protein coupled receptors in the bone, through which it increases osteoblast proliferation and differentiation (Nishida *et al,* 1994). Expression of the parathyroid hormone receptor (PTHR1) has been found to increase with osteoblastic differentiation (McCauley *et al*, 1995, McCauley *et al*, 1996), being first detected in the osteoprogenitor population coincident with or shortly after, the upregulation of AP activity (Rouleau *et al*, 1988, Rouleau *et al*, 1990).

1.7.5 Bone sialoprotein

Bone sialoprotein (BSP) is an acidic glycoprotein which contains an arginine-glycine-aspartic acid (RGD) integrin binding motif (Oldberg *et al*, 1986). It is present in the mineralised matrix and in bone its expression is restricted to osteoid and almost exclusively produced by osteoblasts, osteocytes and hypertrophic chondrocytes (Bianco *et al*, 1993). *In vivo* it is first expressed coincident with the onset of mineralisation (Bianco *et al,* 1991), and in human bone the expression of BSP has been localised to mature osteoblasts (Aubin *et al*, 1993). BSP supports the attachment of osteoclasts and may modulate their activity (Miyauchi *et al*, 1991).

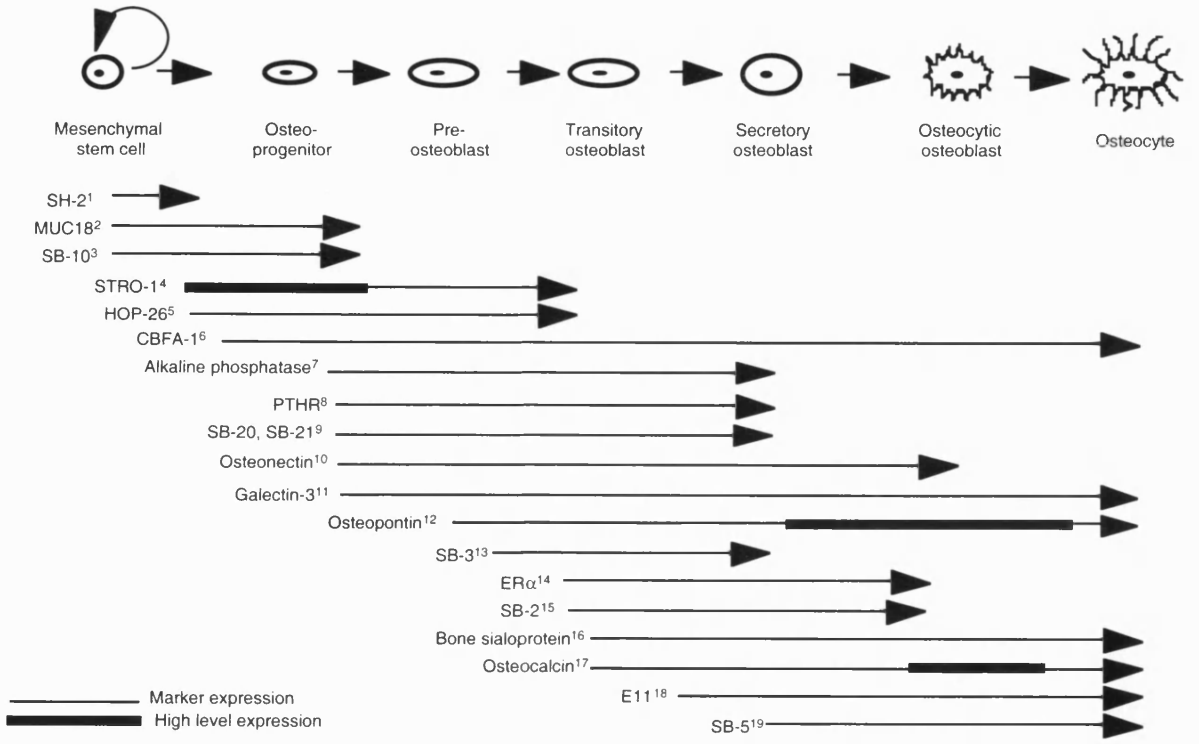


Figure 1.7 The proposed lineage markers for cells of the osteoblastic lineage (adapted from Bruder et al, 1997)

A diagrammatical representation of the osteogenic cell lineage. Expression of selected cell surface and extracellular matrix molecules reported by various investigators. The beginning of each arrow reflects the stage of differentiation where expression is first detected, and the arrowheads show the point where expression is no longer detected. Original references: 1, Haynesworth *et al*, 1992, 2, Filshie *et al*, 1998, 3, Bruder *et al*, 1998, 4, Stewart *et al*, 1996, 5, Joyner *et al,* 1997, 6, Ducy *et al*, 1997, 7, Bruder and Caplan, 1990, Turksen and Aubin, 1992, 8, Rouleau *et al*, 1990, McCauley *et al,* 1996, 9, Bruder *et al*, 1997, 10, Bianco *et al*, 1988, 11, Aubin *et al*, 1996, 12, Mark *et al*, 1987, 13, Bruder and Caplan, 1990a, 14, Bodine *et al*, 1998, 15, Bruder and Caplan, 1989, 16, Chen *et al*, 1991, 17, Bronckers *et al,* 1987, Mark *et al,* 1987, 18, Wetterwald *et al*, 1996, 19, Bruder and Caplan, 1990a.

1.7.6 Oestrogen receptor

For many years the effects of oestrogens on bone were thought to be indirect, but it is now known that low levels of the oestrogen receptor alpha (ERα) are expressed by cells of the osteoblast lineage *in vivo* and *in vitro* (Bodine *et al*, 1998, Hoyland *et al*, 1997). *In vitro*, expression of the ERα is first detected coincident with the upregulation of AP activity. Thereafter its levels increase with further maturation reaching a peak in the osteoblast population and decreasing thereafter (Bodine *et al*, 1998). The expression of high levels of ERα mRNA and protein has also been detected in osteoblasts *in situ*. It has recently been determined that there also exists a β type receptor (Mosselman *et al*, 1996). Expression of ERβ mRNA has been found to increase with osteoblastic differentiation (Arts *et al*, 1997).

1.7.7 Osteocalcin

Osteocalcin (OC), also called bone GLA protein, is a small (7 kDa) noncollagenous protein and is the most abundant non-collagenous protein of the bone extracellular matrix constituting 10%-20% of the total non-collagenous protein (Robey *et al*, 1993). The distinguishing feature of OC is the presence of three γ-carboxyglutamic acid residues, which are dependent on vitamin K for their carboxylation. The synthesis of osteocalcin is upregulated by 1,25-dihydroxyvitamin D_3 (Markose *et al*, 1990) and glucocorticoids (Shalhoub *et al*, 1998). A fraction is released into the circulation, where it can be measured for clinical analysis of bone formation. Two osteocalcin genes have been isolated from the mouse genome and a third osteocalcin-related gene has also been described (Rahman *et al*, 1993). The human osteocalcin gene is considered to be the most specific osteoblast-related gene (Stein *et al,* 1990, Cheng *et al,* 1996, Weinreb *et al*, 1990). It is produced at the very end stage of bone formation perhaps even after mineralisation, and in man is a marker of the osteoblast-osteocyte transition (Owen *et al,* 1990, Pockwinse *et al*, 1992, Kasai *et al*, 1994). Recently, OC has been shown to be a determinant of bone formation in OC deficient mice which have a higher bone mass and bones of improved functional quality (Ducy *et al,* 1996) with an observed increase in bone formation, but no impairment of bone resorption.

1.7.8 Other markers for human cells of the osteoblast lineage

Many other markers of osteoblast differentiation exist, but three which have recently been found are MUC18, SB10 and HOP-26. MUC18 (CD146) is present on freshly isolated bone marrow cells (Filshie *et al*, 1998). It recognises a subset of cells that co-express STRO-1 and contains essentially all assayable CFU-F *in vitro*. However, this antigen is widely expressed on other cell types and is therefore likely to have only limited utility as stage specific marker of osteoblast differentiation. SB10 reacts with an epitope expressed by mesenchymal stromal cells and osteoprogenitors (Bruder *et al*, 1997). The antigen recognised by SB10 has recently been cloned and demonstrated to be activated leukocyte cell adhesion marker type 1 (ALCAM-1/CD166, Bruder *et al*, 1998). HOP-26 (Joyner *et al*, 1997) is strongly reactive with cells in marrow stromal colonies at an early stage of differentiation, prior to the induction of AP expression.

1.8 *Ex-vivo* expansion of CFU-F

Cells within the fibroblastic compartment of bone marrow stroma constitute a potential source of mesenchymal stem cells. *Ex-vivo* expansion of mesenchymal cells by isolation and mitotic expansion of human bone marrow stromal cells *in vitro* could provide autologous tissue of mesenchymal origin for therapeutic treatment of many medical conditions. For example, production of monolayers of mesenchymal cells could be used as a source of grafts for burns, muscle in compound fractures, or bone for non-union fractures or fusion of joints. Culture conditions could be adapted to provide cellular matter to replace necrotic tissue, tissue lost to surgical resection, or for use in reconstructive surgery. Expanded stromal cells could also be used as a delivery system for gene therapy.

The expansion of primitive marrow stromal cells whilst maintaining their maximum developmental potential would allow for the systemic administration of immature cells which might home to bone. If successful, this would result in long term reconstitution of the precursor cell populations, from which the patients would have a renewed cache of precursor cells for the different marrow stromal cell lineages, and progenitor cells could be recruited as necessary. Autologous therapy of this kind has recently been proposed for the treatment of certain types of osteogenesis imperfecta (Marini and Gerba 1997), and may even be applicable to the treatment of osteoporosis.

1.9 Experimental work and aims

Understanding the cause(s) of the decline in osteoblast numbers *in vivo* would represent a significant advance in the prevention of bone loss and, of equal importance, to restore bone mass in patients presenting with established osteoporosis. This would also contribute to the development of new therapeutic regimens that could stimulate bone formation and restore the structural integrity of the skeleton. Additionally, an improved understanding of the factors which regulate normal bone turnover, in particular the proliferation and differentiation of osteogenic precursors, is essential for effective management of many bone diseases and for the treatment of bone defects and inherited disorders using autologous cell therapy.

Methods have previously been described for the isolation of colony forming units-fibroblastic from human bone marrow by density gradient centrifugation and adherence to plastic (Gartner and Kaplan, 1980, Long *et al*, 1990). The CFU-F assay provides a quantitative measure of a compartment of relatively undifferentiated stromal precursors. The purpose of this investigation was to identify factors that influence colony formation and the proliferation of adult human marrow stromal cells *in vitro* with particular emphasis on cells of the osteoblastic lineage.

The specific aims were as follows:

- to attempt to improve the CFE and proliferation of human marrow cells by manipulating the isolation procedures and conditions of culture.

- to determine the influence of PGE_2 on the recruitment of CFU-F from the non-adherent population of cultured BMSC.

- to determine the effect of PDGF isoforms on CFE, proliferation and the osteogenic differentiation of BMSC.

- to determine the influence of age and gender on CFE, proliferation and the osteogenic differentiation of BMSC.

- to attempt the generation of immortalised strains of BMSC by transfection with plasmid expressing SV40 large T antigen under the control of an inducible promoter.

Chapter 2

Materials and Methods

2.1 Materials and reagents

2.1.1 Human bone marrow samples

Samples were obtained from the Bristol Royal Infirmary and Frenchay Hospital, Bristol. Adult human bone fragments were collected from the operating theatre within 4 hours of surgery. Samples were most commonly rib from patients undergoing cardio-thoracic surgery, only bone which would otherwise have been discarded was used and its use was approved by the Local Ethics Committee. Selection criteria excluded bone from patients who were suffering underlying bone disease or being prescribed corticosteroids. For each patient the age, sex, recent drug history, underlying pathology and site of skeletal origin was recorded (the characteristics of samples used in this study are shown in appendix III). For shipment to the laboratory the bone was placed into sterile flasks, submerged in sterile Dulbecco's modified Eagle's medium (DMEM) and delivered via courier. Bone was received and processed within 8 hours of its removal from the patient.

2.1.2 Use of cell lines

Mesenchymal-derived cell lines were utilised to evaluate methods or provide control samples. The selected cells lines used were: MG-63 cells (ATCC, CRL 1427), $MRC_5SV_1TG_1$ cells (ECACC #85042501), and Chinese hamster ovary cells (CHO, ECACC #5050302). MG-63 are a human osteosarcoma cell-line which express some of the characteristics of osteoblast-like cells isolated from the surface of human trabecular bone. $MRC_5SV_1TG_1$ is an SV40 transformed human foetal lung cell-line which has a fibroblastic morphology and expresses SV40 large T antigen. CHO cells have an epithelial morphology and were used for optimising stable transfection techniques.

2.1.3 Handling and culture of cellular material

Tissue preparation, cell culture and work requiring sterile conditions was carried out in a Microflow Class 2 Biological Safety Cabinet, MDH, U.K. The cabinet was cleaned with neat Ambisan solution (Deb Ltd., U.K.) and 70% ethanol (Hayman Ltd., U.K.) once a month. The incubator used was a CO_2 incubator (Borolabs, U.K.). Cells were cultured at 37°C in a humidified atmosphere of 95% air : 5% carbon dioxide.

2.1.4 Plastics

All tissue culture plastic disposables were obtained from Becton Dickinson Labwear, N.J.

2.1.5 Reagents

Unless stated otherwise all reagents were prepared in ultra pure water (Milli-Q Water System, reagent grade, Millipore (U.K.) Ltd.)

2.1.5.1 General laboratory chemicals

All chemical reagents were purchased from Sigma Chemical or BDH Ltd. unless otherwise indicated, and were of molecular or AnalaR grade.

2.1.5.2 Tissue culture reagents

These were supplied by Gibco BRL Life Technologies, U.K. unless stated otherwise.

2.1.5.3 Growth factors and inhibitors

Recombinant human platelet-derived growth factor (PDGF)-BB and AA were obtained from R & D Systems, and resuspended in 4 mM hydrochloric acid (HCl) containing 0.1% bovine serum albumin (BSA).

Recombinant human αIGF-I (R & D Systems) was dissolved in 10 mM acetic acid with 0.1% BSA.

Anti-IGF (αIR-3) antibody was obtained from Dr F. C. Kull, Glaxo Welcome Inc., USA.

PGE_2 stored under ethanol was a kind gift from Dr A. Scutt, University of Sheffield.

2.1.5.4 Antibodies

Hybridomas for the monoclonal mouse anti-human antibodies, STRO-1 and AP (B4-78) were obtained from Dr. P. Simmons, University of Adelaide, Australia and the Developmental Studies Hybridoma Bank, University of Iowa (Simmons and Torok-Storb, 1991 and Lanson *et al*, 1985 respectively). These were cultured and the supernatants containing secreted antibody collected for use. The AP antibody was purified by protein G column separation by Dr. Carolyn Jefferiss. IgG1 was supplied by Sigma, and OB/L was raised in-house by Dr. Susan Walsh. The goat anti-mouse secondary conjugates, anti-IgG1 RPE and anti-IgM FITC were supplied by Southern Biotechnology Associates Inc.

Rabbit polyclonal IgGs against PDGF receptor α chain (C-20, #sc-338) and PDGF receptor β chain (958, #sc-432) were obtained from Santa Cruz Biotechnology Inc. These were specific for epitopes corresponding to amino acids 1065-1084 and 958-1106 mapping at the carboxy terminal domain of the precursor form of human PDGF receptor type α and β respectively.

1.5 mg/ml pAb101 mouse IgG2α, isotype specific for the human C-terminal of SV40 large T antigen, was obtained from ATCC #TIB 117 and prepared in DMEM with 20% FCS. The antibody was used at a concentration of 5 μg/ml.

2.1.5.5 Enzymes

Collagenase VII (Sigma) was made up in DMEM with 5 mM $CaCl_2$, and stored in 1 ml aliquots of 2500 U/ml at -20°C for up to 1 year.

Trypsin-EDTA was a 1 x stock solution of 0.5g Trypsin, 0.2g EDTA, 0.85g NaCl per litre, from Gibco BRL.

Superscript ™ RNase H^- Reverse Transcriptase was supplied at 200 U/µl by Gibco BRL

Taq DNA Polymerase was supplied in storage buffer A, at 5 units/µl (Promega #M1861).

2.1.5.6 cDNA primers

The following primers were used in preparation of cDNA:

Target cDNA	Primer sequence (5'-3')	Amplimer size (bp)
GAPDH	5': GGTGAAGGTCGGAGTCAACGG	570
	3': TAGCACCTTCCTGAGTACTGG	
PTHR	5': AGAAGAAGTACCTGTGGGGCTTCACAG	275
	3': GCTTGCGGTACTGCTGCCGTGTGTCAC	
BSP	5': GCAGCCGGATCCTCATGCATTGGCTCCAGTGACACT	700
	3': GGAATTCTGCTCAGCATTTTGGGAAT	
OC	5': ATGAGAGCCCTCACACTCCTCG	285
	3': CTAGACCGGGCCGTAGAAGCG	
ER	5': CAGTGAAGCTTCGATGATGATGG	300
	3': TACTACATCGGTCGTCGTAC	
PDGF A	5': AGAAGTCCAGGTGAGGTTAGAGGAGCAT	304
	3': CTGCTTCACCGAGTGCTACAATACTTGCT	
PDGF B	5': GAAGGAGCCTGGGTTCCCTG	217
	3': TTTCTCACCTGGACAGGTCG	

2.1.5.7 Radioisotopes

[^{3}H]-thymidine was obtained from ICN, Ltd. U.K. at 37 kBq /µl in aqueous solution.

[^{32}P]-adenosine 5'-triphosphate was supplied in aqueous solution at 370 kBq/µl by Amersham Life Sciences, U.K. Ltd.

2.1.5.8 Solutions

The preparation of solutions described in these methods are shown in appendix I.

2.2 Tissue Culture

2.2.1 Isolation and culture of human bone-derived cells

2.2.1.1 Bone marrow stromal cells (BMSC) and marrow cell populations

Human bone was submerged in Dulbecco's modified Eagle's medium (DMEM) in a petri-dish and the periosteum and all other extraneous connective tissue were removed by scraping with a sterile scalpel blade. Once clean, the bone was placed into fresh medium and split open lengthways with bone cutters. Fragments of bone, and the DMEM containing marrow tissue were transferred to a sterile 50 ml polypropylene tube (Falcon, Becton Dickinson). Marrow tissue was liberated from the rib by vortexing the sample in at least three volumes of DMEM until the bone was blanched. After each wash the supernatant was decanted into a fresh tube. An aliquot of the extracted marrow was placed aside and termed a whole marrow (WM) sample. The marrow cells were then pelleted by centrifugation at 514 x g for 5 mins in a Beckman GPR Centrifuge (rotor GH 3.7, Beckman, U.K). The fatty layer was aspirated off and the supernatant removed and retained as a subpopulation for further investigation, termed the 'wash'. The marrow cells were then resuspended in 25 ml DMEM, layered over 20 ml Lymphoprep™ (1.077 g/ml) and subjected to equilibrium-density centrifugation for 30 mins at 913 x g with the centrifuge brake off. The mononuclear cells were collected by transferring the opaque interface into a fresh tube whereupon 30 ml of DMEM were added and the cells spun again at 1430 x g for 10 mins to wash out any remaining Lymphoprep. This isolate contained bone marrow stromal cells (BMSC). The pellet obtained post-density gradient centrifugation was submitted to a second round of density-gradient centrifugation (double-ficolled). The isolated population of erythrocytes (RBC) were resuspended in serum-free medium.

The recovered marrow cell populations were resuspended in 15 ml of DMEM and filtered through a 70 μm filter (Falcon) to obtain suspensions of single cells. The number of viable mononuclear cells in each population was determined using trypan-blue exclusion and a haemocytometer (section 2.3.2), and using an electronic particle counter (Coulter Counter®, Multisizer II, Coulter Electronics, U.K., section 2.3.1). Each marrow cell population was seeded at $2x10^4$ single cells/cm^2 in petri-dishes or flasks and cultured in standard medium; DMEM supplemented with 15% (v/v) of foetal calf serum (FCS, see appendix I for selected lots), with or without 10^{-4} M L-ascorbate 2-phosphate (ASP) as required. Following 4 days incubation non-adherent cells were aspirated under low vacuum pressure and adherent cells washed with 2 x 5 ml of phosphate buffered saline (PBS). Thereafter, medium was replaced bi-weekly until 18 days when the cultures were fixed, or passaged for further study. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.2.1.2 Human bone-derived cells (HBDC)

The explant procedure followed was essentially that described by Gundle and Beresford (1995). Briefly, following removal of the bone marrow (section 2.2.1.1) trabecular bone was scraped from the endosteal bone surface into PBS and cut into fragments ~3 mm in diameter using a sterile scalpel blade. Fragments of bone were transferred to sterile 50 ml polypropylene tubes and washed by vortexing vigorously in 3 volumes of PBS to remove remaining blood and marrow tissue. Once free of contaminating marrow elements, the fragments of trabecular bone were returned to a petri-dish and covered in PBS. Bone pieces were divided into piles of 1 cm^2 x 0.5 cm. Each pile was placed into a 75 cm^2 flask with 12 ml of standard medium (10% (v/v) FCS in DMEM). The explants were then left undisturbed in an incubator at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was replaced at 7 days, then twice weekly thereafter with care taken to not dislodge the bone fragments. Cellular outgrowths were generally observed at between 7-14 days and cells reached confluence at 4 to 6 weeks.

2.2.1.3 Cell lines

A cryovial containing cells of a cell-line was removed from liquid nitrogen storage and defrosted rapidly at 37°C. 0.5 ml aliquots were transferred to two 75 cm^2 flasks with 15 ml of standard medium (10% FCS in DMEM for MG-63 and $MRC_5SV_1TG_1$ cells). CHO cells were cultured with 10% FCS in glutamine-free DMEM. The contents of the flask were swirled gently and incubated overnight in a humidified incubator at 37°C. The medium was replaced the following day to remove traces of dimethylsulphoxide (DMSO). Thereafter, the cultures were fed every 2-3 days until the cells reached confluence (10-14 days).

2.2.2 Passaging cultured cells

BMSC and HBDC:

Bone marrow and trabecular cultures required pre-incubation with bacterial collagenase to degrade the collagen rich extracellular matrix before digestion with trypsin-EDTA was possible. The protocol employed has been described previously (Gallagher *et al*, 1996). Briefly, the culture medium was aspirated under low vacuum pressure and the cell monolayer washed by addition of 2 x 5 ml serum-free DMEM to remove residual FCS. 50 µl/cm^2 of 25 U/ml collagenase VII in serum-free DMEM supplemented with 2 mM $CaCl_2$ was then added and the cells incubated for one hour at 37°C. At the end of this period, and following examination to ensure that the cells had not detached, the collagenase solution was removed and the cell layer washed twice in PBS.

50 µl/cm^2 1 x trypsin-EDTA (Gibco BRL) was then layered over the cells and the flask incubated at 37°C for 5-10 mins. The process of cellular detachment was observed by microscopy (x 100) throughout subculture. A cell suspension was produced by gently pipetting up and down with a sterile 2 ml pipette to break down large clumps of cells. The suspension was transferred to a fresh 50 ml polypropylene tube containing 1% FCS to neutralise the trypsin activity. The flask was rinsed with 2 ml DMEM and the washings pooled with the cell suspension. Cells were recovered by centrifugation at 514 x g for 5 mins at 4°C. The cell pellet was resuspended in a small volume of DMEM, typically 2 ml per 50 cm^2 monolayer. The resulting cell suspension was filtered through a 70 µm filter (Becton Dickinson) to remove clumped cells and matrix material, then counted electronically (2.3.1). BMSC and HBDC were subcultured at 1x10^4 cells/cm^2.

Cell-lines:

Cell-lines (2.2.1.3) were subcultured when monolayers reached 80% confluence (generally twice weekly) at a split ratio of 1:5. Medium was aspirated and the cell monolayer washed with 2 x 5 ml DMEM. Cells were detached from the substratum by trypsin-EDTA digestion, and the above protocol was followed.

2.2.3 Cryogenic storage of cell-lines

Following passage of cultures, cells were stored to maintain stocks. 5x10^6 cells were resuspended in 0.5 ml freezing medium containing 10% (v/v) DMSO, 50% (v/v) FCS, and 40% (v/v) DMEM. 0.5 ml of cell suspension was placed in a cryovial and slowly frozen through 4°C (30 mins), -20°C (2 hours) and -70°C overnight, before transferring to liquid nitrogen for long term storage.

2.2.4 Redistribution of bone cells and terminology of passaged cultures

The first flask of cells grown from BMSC are referred to as the primary culture, and proliferation at this stage is predominantly clonal. The growth of the colonies is rapid for the first 14-18 days of culture, but slows as the cells become densely packed and exit from the cell-cycle and/or begin to differentiate. To maintain proliferation, cells within the colonies were redistributed after 18 days, with the additional benefit that the yield of cells (after 21-28 days) is increased. Cells were redistributed using sequential collagenase-trypsin-EDTA treatment described previously (section 2.2.2). Standard medium was added to the cells which were left to resettle and grow for 14 days. Cultures treated in this way are termed redistributed primary cultures. If many cells were required, redistributed cultures were passaged after a further 14 days, seeded at 1x10^4 cells/cm^2 (passage 1), and then grown until 75% confluent. They were not passaged further.

2.3 Counting cells

2.3.1 Electronic particle counting

A Coulter Counter detects particles of a defined size suspended in an electrolyte passing through an aperture over which there is a potential difference. The particles in the aperture displace electrolyte and alter the resistance between electrodes. The method has advantages over the haemocytometer method as the counter determines the number of cells electronically (preventing human error), is 40 times more sensitive (i.e. it can detect as few as 1250 cells per ml) and can be attenuated to count cells of a certain size range. It does not, however distinguish viable from dead cells. 200 µl of cell suspension was mixed with 10 ml azide-free isotonic buffer (Isoton® II, Coulter Euro Diagnostics GMBH) in a 20 ml vial (Coulter) and the number of cells in 1 ml of suspension were determined electronically (Coulter Counter®, Multisizer II, Coulter Electronics, U.K.). The total number of cells in suspension was calculated as the number of events in 1 ml Isoton suspension, corrected for the dilution factor (10.2 ml/ 0.2 ml = 51), times the volume of the original suspension (ml).

2.3.2 Measurement of cell viability

The number of viable cells in a cell suspension (excluding freshly isolated bone marrow cell suspensions) was determined by mixing 10 µl cell suspension, 10 µl trypan blue (0.5% solution in 85% saline, Flow Laboratories Inc.) and 30 µl serum-free DMEM. The mixture was allowed to stand for 2 mins, then 15 µl were loaded onto a haemocytometer and counted. Exclusion of trypan blue and a highly refractive appearance indicated cellular viability. Viable cells lying within the haemocytometer grid were counted, including cells lying to the left vertically or the top horizontally. A count was taken until at least 50 viable cells had been counted. This number was multiplied by 10,000 to account for the dimensions of the haemocytometer, and again by 5 to compensate for dilution.

To count nucleated cells in freshly isolated bone marrow stromal cell preparations, a 3% (v/v) acetic acid solution in water stained with 0.01% (w/v) methylene blue was mixed 1:10 with a cell suspension. This stains all nucleated cells and destroys red blood cells. 15 µl were loaded onto a haemocytometer and counted as above.

2.4 Experiments on primary cell cultures

For each treatment group in an experiment the following parameters were determined, i) total number of colonies, ii) proportion of total colonies expressing alkaline phosphatase, iii) the mean size of the colonies and, iv) the total number of cells per flask. Each treatment was carried out in quadruplicate. Unless stated otherwise the test factors were added to the cells at the time of explantation, and maintained in culture until analysis.

2.4.1 Determination of colony forming efficiency (CFE)

BMSC were prepared and cultured in 55 mm diameter petri-dishes as described in section 2.2.1.1. At 18 days post-explantation the medium was aspirated, and the cells washed in two volumes of PBS. Colonies were fixed in a solution of 10% (v/v) neutral buffered formalin (NBF) for 15 mins at 4°C and then stained sequentially to identify colonies of fibroblastic or osteoblastic cells. Cellular foci containing > 50 cells were defined as colonies. Foci containing < 50 cells were defined as clusters and not scored. Use of a 15 x objective (microscope model SZ-CTV, Olympus, Japan) aided counting. Mean area per colony was determined by measuring the colony diameter with a ruler and calculating the area using πr^2. The area of all colonies in a dish was totalled and divided by the number of colonies.

2.4.2 Expression of alkaline phosphatase (Dakopatts protocol, Denmark)

Substrate solution was prepared by dissolving naphthol AS-MX phosphate in N,N-dimethylformamide at 10 mg/ml in a glass tube, and diluting 1 in 50 with 0.1 M Tris buffer, pH 8.2. Fast-red TR salt (1 mg/ml) was dissolved in the substrate solution and filtered (paper #1, Whatman) immediately prior to use. Colonies were covered with 2 ml of Fast-red TR solution and incubated for 30 mins at 37°C. The stain was rinsed off with tap water and the dishes air dried. Colonies were scored as AP positive if greater than 20 % of the cells were stained with Fast-red TR.

2.4.3 Demonstration of mineral deposition

The presence of deposited mineral was detected using von Kossa reagent. The method is based on the substitution of calcium bound to phosphate and carbonate groups by silver ions. A method modified by Rungby *et al* (1993) was followed to reduce background staining. Colonies were cultured from fresh human bone marrow stromal cell explants as previously described (2.2.1).

To facilitate matrix mineralisation, 5 mM inorganic phosphate (Pi) was added to standard medium on day 12 for 5 days. Following aspiration of the medium, colonies were rinsed once with PBS and then fixed in 10% NBF for 15 mins at 4°C. The fixative was removed and the cultures rinsed with distilled water for 1 x 1 minute, and 1 x 15 mins. The colonies were stained with a solution of 0.5% (w/v) silver lactate for 20 mins in daylight. The stain was rinsed away with distilled water and the colonies then immersed in 0.5% (w/v) hydroquinone for 2 mins, and 5% (w/v) sodium thiosulphate for 2 mins. The dishes were rinsed twice in distilled water and the cells counter stained with 0.1% (w/v) toluidine blue O for 2 seconds. The staining solution was washed away with 3 volumes of tap water and the dishes air dried. Mineralisation was indicated by the presence of a black deposit on or surrounding fixed cells.

2.4.4 Methylene blue stain of total BMSC colonies

Visualisation of total colonies was facilitated histochemically by staining fixed cells with methylene blue. Following AP and/or von Kossa stain, fixed cultures were washed with borate buffer (10 mM, pH 8.8), stained with 1% (w/v) methylene blue solution prepared in borate buffer for 3 mins, then rewashed with 3 volumes of borate buffer. The dishes were air dried and the methylene blue positive colonies were counted as total colony number. As an indirect estimate of total cell number, methylene blue was eluted and the OD measured at 650 nm (Currie, 1981). This was achieved by eluting dye with 500 µl of 1% HCl in ethanol. The dishes were rinsed in a further 500 µl acidified ethanol and the volumes pooled. 50µl of eluate was transferred to a 96 well microtitre plate and the absorbance read in a Dynatech MR5000 scanning multiwell spectrophotometer at 650 nm against acidified ethanol blanks.

2.5 Cell proliferation experiments

Cell proliferation was expressed as the mean number of cells per cm^2 for n = 4 flasks. Following 28 days incubation the culture medium was aspirated and the cells washed in two volumes of PBS. Cells were detached from the plastic by sequential collagenase, trypsin-EDTA treatment (section 2.2.2), collected by centrifugation (514 x g for 5 mins) and resuspended in 2 ml of medium. The cell suspension was filtered and the number of cells in suspension determined electronically using a Coulter Counter (section 2.3.1).

2.5.1 Tritiated thymidine incorporation and scintillation counting

Cells were harvested, washed in medium and resuspended at a concentration of $1x10^6$ cells/ml. The cells were then aliquotted into wells of a 96-well flat bottomed plate ($1x10^5$ cells/well) along with the factors being investigated. DNA synthesis was measured through incorporation of [^{3}H]-thymidine. Cells were incubated in 200 µl medium at 37°C in 95% air, 5% CO_2 for 72 hours in the presence of test factors and then pulsed with [^{3}H]-thymidine. Stock [^{3}H]-thymidine (1 µCi/µl) was diluted 1 in 50 in medium. 25 µl of diluted [^{3}H]-thymidine were added to the cells which were incubated at 37°C for a further 24 hours. The medium was aspirated and replaced with 100 µl of 0.1% SDS in H_2O. The cell lysate was harvested onto glass filters using a semiautomatic cell harvester (Filtermate 196, Packard). The filters were dried, and immersed in 500 µl of Optiscint HiSafe in scintillation vials. The amount of incorporated [^{3}H]-thymidine was measured as scintillation counts per minute (CPM) on a LKB 1209 Rackbeta liquid scintillation counter.

2.6 The effect of growth factors on osteoblastic differentiation

2.6.1 Preparation of cell protein extracts

Protein lysates were used for biochemical analysis of alkaline phosphatase, dot blots and western blot studies. Bone-derived cells were harvested at 75% confluence as described in section 2.2.2. $1x10^6$ cells were aliquotted into sterile universal tubes and recovered by centrifugation at 514 x g for 5 mins. The cell pellet was washed in two volumes of PBS with added protease inhibitors, then resuspended in 100 µl of ice-cold Nonidet P-40 buffer (NP-40) + inhibitors. The cell suspension was transferred to a cooled microcentrifuge tube (Sigma). The suspension was left on ice for one hour to lyse the cells. The homogenate was then centrifuged for 10 mins at 10,000 x g in a bench-top centrifuge (4°C, Biofuge Fresco, Heraeus Instruments) to pellet the cell nuclei and insoluble material. The supernatant containing soluble proteins was placed in a fresh microcentrifuge tube and stored at -70°C.

2.6.2 Total protein determination in cell lysates

The DC Protein Assay (Biorad) was used for the determination of protein concentration in cell lysates. The assay is compatible with lysis buffers containing detergents (NP-40) and protease inhibitors. Protein dilutions of 0 - 3 mg/ml bovine γ-globulin protein were prepared from a 20 mg/ml stock solution diluted in the buffer in which the samples were resuspended. Standards and samples (5 µl) were pipetted into wells of a clean dry microtitre plate and 25 µl of mixed Reagent A (sodium hydroxide, NaOH) was added to each well. If detergent was not present in the sample buffer, Reagent S (SDS, 20µl) was added to each ml of Reagent A required. 200 µl reagent B (Folin Reagent) was then added and the plate agitated to mix the reagents. After 15 mins the A750 nm was determined using an MR5000 plate reader (Dynatech) and the protein concentrations of the samples determined from the standard curve.

2.6.3 Biochemical analysis of alkaline phosphatase activity

Alkaline phosphatase activity in whole cell lysates was determined by measuring the formation of yellow p-nitrophenol (p-NP) by hydrolysis of colourless p-nitrophenyl phosphate (p-NPP) substrate at pH 10.3 for 30 mins at 37°C. Lysates of $1x10^6$ cells were prepared as in section 2.6.1, although, cell pellets were resuspended in 500 µl PBS.

A standard curve (range 10 nmol/ml - 2 μmol/ml) was prepared by diluting 10 μmol/ml p-NP standard solution in 221-Alkaline buffer solution (Sigma). 25 μl of standards and samples (equivalent to $5x10^4$ cells) were transferred to triplicate wells in a microtitre plate. Substrate buffer was made up by dissolving p-NPP (460 μg/ml) in 221-Alkaline buffer supplemented with 0.5% (v/v) Triton-X. 200 μl substrate buffer was added to each standard and sample well. The plates were incubated at 37°C for 30 mins and then the reaction stopped by adding 20 μl of 10 M NaOH. The optical density equivalent to [p-NP] was read at 410 nm against blanks of substrate buffer in an MR5000 plate reader (Dynatech) and the concentration of p-NP calculated automatically from the standard curve. The results are expressed as nmole of p-NP produced per $1x10^6$ cells per minute.

2.6.4 1,25-Dihydroxyvitamin D_3 pulse in serum-free medium

To increase the synthesis and secretion of osteocalcin in osteoblastic cells, culture medium was aspirated from primary cells, the monolayer rinsed with 3 volumes of PBS to remove any Dx present in the culture. Medium was replaced under UV-free conditions with serum-free medium containing 1,25-dihydroxyvitamin D_3 (13 μg/ml stock, gift from Roche, Welwyn Garden City) and vitamin K both at a final concentration of 10^{-8} M for 48 hours.

2.6.5 Osteocalcin assay

After 48 hours treatment with 1,25-dihydroxyvitamin D_3, 1 ml of culture supernatant was harvested and snap frozen in liquid nitrogen. All samples were stored at -70°C and were subjected to fewer than two freeze-thaw cycles. Osteocalcin levels in the culture supernatant was determined using a competitive immunoassay, specific for the intact protein (Novocalcin, Metra Biosystems Inc.). Before use, all reagents were warmed to room temperature. 25 μl of standards, samples and controls were added to osteocalcin coated strips of a microtitre plate and 125 μl of osteocalcin antiserum were added. The plate was gently swirled for 1 minute then tightly covered and incubated at room temperature for 2 hours. Following incubation the plate was inverted and washed with 3 x 300 μl volumes of 1 x wash buffer.

150 μl of reconstituted enzyme conjugate were added to each well and then incubated at room temperature for 1 hour. The strips were washed again, blotted dry and 150 μl of working substrate solution was added to each well. The plate was incubated for a further 35 mins at room temperature, then the reaction stopped with 50 μl of 3M NaOH. The optical density was read within 15 mins of NaOH addition at 405 nm on an MR5000 plate reader (Dynatech), and absorbances compared with a 4-parameter calibration curve fitting equation.

2.6.6 Cyclic AMP detection

BMSC in 2° culture were grown to 70% confluence. The medium was removed and replaced with medium containing a range of [PGE_2]. Cultures were treated for 15 mins. The medium was then diluted with 2 volumes of absolute ethanol at -20 °C, and transferred to a centrifuge tube. The medium was evaporated on a rotary evaporator (Jouan RC. 10.22) for 350 mins at 50°C. The residue was resuspended in assay buffer, and the amount of cyclic adenosine monophosphate (cAMP) present was measured using a commercially available kit (Biotrak, cAMP EIA, Amersham Life Science). [cAMP] was normalised for the number of cells in the culture dish determined indirectly using the dye methylene blue (section 2.4.4).

2.7 Statistical analysis

CFE and cell number were determined using a minimum of 4 cultures and the data expressed either as the mean +/- SE or as the treatment/control (T/C) ratio, with the absolute values observed in the control groups stated in the text. Where experiments were performed with multiple donors and similar results were generated, summary data are expressed as percentages of control values and errors are equivalent to standard errors between the donor samples. Statistical analysis was carried out on all data shown.

2.7.1 Kruskal-Wallis test

No assumptions were made regarding the underlying distribution (parametric vs non-parametric) of the individual data points of colony formation or proliferation. For this reason statistical analysis of the data was performed using the Kruskal-Wallis test. This can be used to analyse more than three sample groups of varying sizes and, as it is based on a ranking system and compares the median values of the samples, the absolute magnitudes of the changes in parameter are not considered, merely whether or not a change was observed following treatment with one or more agent.

The H value is used to compare the median values between all the sample groups tested. A value determined experimentally shown to be equal to, or greater than the critical H value indicates a statistically significant result (Neave, 1981). H was calculated by the formula:

$$H=[12/N(N+1)]\sum_{1}^{k}(R^2/n)-3(N+1)$$

where N = total number of values analysed, k = the number of samples, Σ = sum of, R = rank of each sample, and n = size of each sample (number of repeats per treatment).

The second stage of the Kruskal-Wallis test is to compare samples with each other by use of Q values. It is noted however, that testing for the value of Q was only performed where a statistically significant H value was obtained. Q values determine the statistical significance of the difference observed between individual sample medians and, as for H values, an experimentally determined value equal to, or greater than the critical Q value is indicative of statistical significance (Heath, 1995). The Q value is calculated by the formula:

$$Q = \frac{R1 - R2}{\sqrt{N\frac{(N+1)}{12}X\left(\frac{1}{n1}+\frac{1}{n2}\right)}}$$

where R = mean rank of sample, N = total numbers of values analysed in the Kruskal-Wallis test, n = size of each sample (number of repeats per treatment). Significant results are stated in the text.

2.7.2 Spearmans rank correlation coefficient

The effect of age on each parameter was assessed using Spearmans rank correlation coefficient calculated using a computer package (SRCC, StatView v4.51). This is a non-parametric test which has few underlying assumptions of means and variance. The test ranks the two sets of data independently (in this case, age and colony formation or cell number) and calculates the correlation coefficient (r) using the formula below:

$$rs = 1 - \frac{6\sum d^2}{n^3 - n}$$

where rs = Spearmans rho, the calculated value of Spearmans rank correlation coefficient, d = the difference between the ranks of the two variables for any sampling unit (d is small with a positive correlation, and large with a negative correlation), and n = the number of samples. rho tests for the tendency of Y to increase (or decrease) as X increases on a bivariate plot, and varies between +1 for a perfect positive correlation and -1 for a perfect negative correlation, the sign indicating the direction of correlation. The minimum number of patient samples assessable is five.

The Spearman rank correlation coefficient uses the null hypothesis that there is no correlation between the two variables. The general null hypothesis used was that there was not an association between donor age and the parameter under investigation. The calculated value of rho was compared to the critical value at a confidence level of 5%. A two tailed analysis ($\alpha 2$) was used to avoid assumption of the direction of correlation, and a one tailed analysis ($\alpha 1$) was used when the direction of correlation could be assumed from a plot.

The null hypothesis was rejected if the calculated value was equal to, or larger than, the critical value. The results are shown in appendix II, where the p-value in the analysis tables shows the associated probability derived from a comparison of the calculated value with the critical value for the sample number, n. It was decided that correlations equating to $p < 0.05$ showed significance and the null hypothesis was rejected.

2.7.3 Mann-Whitney U test

The Mann-Whitney U test is a non-parametric version of the two group unpaired t-test, and tests the hypothesis that two populations have the same median (Heath, 1995a). Since the Mann-Whitney U test does not consider the observations but instead their ranks, it is resistant to outliers in either of the groups compared. The requirements for validity of the Mann-Whitney test are that the two groups of observations come from continuous distributions and are independent of each other. This analysis was calculated using the StatView, v4.51 computer package. The U value was calculated as follows. All the samples are ordered and ranked as one group. The sum of the ranks for A or B are calculated (R_A or R_B), then U_A or U_B are obtained as below (for A):

$$U_A = R_A - \frac{1}{2} n_A(n_A + 1)$$

where, n = the sample size. U is then the smaller of U_A or $n_A n_B$ - U_A and the critical value of U is less than the tabulated value.

2.8 Immunocytochemistry

2.8.1 Immunocytochemistry of $MRC_5SV_1TG_1$ cells

Cytospins of $MRC_5SV_1TG_1$ cells were prepared to investigate expression of the simian virus 40 large T antigen (SV40 large T Ag) by immunocytochemistry. 200 µl of cell suspension ($2x10^5$ cells) was placed in the Cytofunnel™ sample chamber, clipped onto the microscope slide against a filter card with a Cytoclip™ and centrifuged at 55 x g for 3 mins in the Shandon Cytospin 3. The slides were allowed to air dry and then fixed in acetone for 5 mins at 25°C. Following evaporation of the acetone, a ring was drawn around the cells using a Pap pen. Slides could be stored wrapped in foil at -20°C.

Monoclonal antibody, pAb101 (100 µl of 5 µg/ml in PBS, IgG2α) was placed on the cells which was isotype specific for the C-terminal of SV40 large T Ag. The antibody was incubated on the slides for 30 mins, in a petri-dish containing moistened filter paper to prevent drying. Unbound antibody was rinsed off the slides with PBS, 1% FCS in a Coplin jar. The secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody directed against the γ-chain of IgG was added (1 in 75 dilution, 200 µl), and incubated at 25°C for 30 mins. The 2° antibody was washed off in PBS, 10% FCS. The cells were counter-stained with 0.1% ethidium bromide and washed thoroughly. One drop of mountant (1:1 mixture of glycerol and PBS) was placed on the labelled cells and a cover slip layered over the mountant avoiding incorporation of air bubbles. The slides were viewed under a light microscope (100 x objective) with a UV lamp (302 nm).

2.8.2 Detection of cell surface antigens

Two sets of experiments were carried out using fluorescent activated cell scanning (FACS®) to examine the presence of specific epitopes on the cell surface. These were i) intracellular labelling with antibodies directed against the PDGF α or β receptor types, and ii) dual surface labelling with antibodies against STRO-1 and AP epitopes.

Redistributed BMSC and trabecular bone cells were cultured in the absence or presence of test factors. At 75% confluence the cell monolayer was harvested by sequential treatment with collagenase-trypsin (section 2.2.2) and resuspended in DMEM supplemented with 1% FCS. Cells were incubated at 37°C for 30 mins to allow recovery after trypsin treatment. At this stage the cells were counted using a Coulter Counter (section 2.3.1). The cells were then centrifuged at 514 x g for 4 mins at 4°C, the supernatant removed by aspiration and the cells resuspended in 3 ml of FACS wash buffer. This washing process was repeated twice. The cells were finally resuspended at $1x10^6$ cells/ml in wash buffer. 500 µl of cell suspension ($5x10^5$ cells) were aliquotted into polystyrene FACS tubes (Falcon, Becton Dickinson). The aliquots were kept on ice during the following incubation steps.

2.8.3 Flow cytometric analysis of STRO-1 and alkaline phosphatase

Aliquotted cells were pelleted, and resuspended in blocking buffer (50 µl per tube) and incubated for 30 mins to block Fc receptors on the cell surface. The cells were washed in wash buffer and then incubated for 45 mins with STRO-1 hybridoma culture supernatant (500 µl per tube) containing the purified anti-alkaline phosphatase antibody, B4-78, at 15 µg/ml.

For cells from each treatment group the following tubes were set up:

Tube	Ab combination
Negative	IgM + IgG1
STRO-1+	STRO-1 mAb + IgG1
AP+	AP mAb + IgM
Dual positive	STRO-1 mAb + AP mAb

After incubation the cells were washed once in 3 ml wash buffer and then resuspended in 100 µl FITC-conjugated and RPE-conjugated 2° antibodies combined in blocking buffer. The cells were incubated for 45 mins then washed once in 3 ml wash buffer. Labelled cells were finally resuspended in 200 µl PBS with 200 µl FACS-fix and stored in the dark for up to 4 days at 4°C prior to analysis.

2.8.4 PDGF receptor expression

A rabbit anti-human PDGF receptor antibody directed against an intracellular epitope, was used to examine receptor expression on bone-derived cells. Cells were permeabilised with saponin during incubation with the antibody to allow access to the antigenic site.

The cells were fixed initially for 5 mins in 200 µl 2% FACS fix in order to fix the cell membrane prior to it being permeabilised. The fix was rinsed away with 500 µl FACS wash buffer and the cells recovered by centrifugation. The cells were then permeabilised y incubation with 500 µl of saponin solution for 5 mins. The cells were again washed and then incubated for 60 mins in 100 µl of monoclonal rabbit anti-human PDGF receptor antibody diluted to 1 µg/ml in saponin solution. Negative control tubes were set up with isotype control antibody (rabbit IgG at 1µg/ml in saponin solution). Following incubation the cells were washed twice in 2 ml saponin solution, and recovered by centrifugation. The cells were resuspended in 100 µl FITC-conjugated goat anti-rabbit IgG diluted 1 in 160 in FACS wash buffer, and incubated for 30 mins. Finally, the cells were washed once in 2 ml saponin solution, and then in FACS wash buffer to 'reseal' the membranes. Labelled cells were recovered by centrifugation and resuspended in 500 µl of 1% FACS fix diluted in FACS wash buffer and stored in the dark for up to 4 days at 4°C prior to analysis.

2.8.5 Flow cytometry

Samples were analysed on a Becton Dickinson FACStar Plus, equipped with a 100 mW argon ion laser and controlled by a Consort 32 computer. The principle of flow cytometry is that a single cell suspension passes through a laser light source which causes any bound fluorochrome to emit light of a different wavelength. The scattered or fluorescent light produced is collected by a photo multiplier tube (PMT), which is converted to electrical signals and eventually to digital information. The light produced is reflected by mirrors, coated such that they only reflect light of a specific wavelength. Therefore, one cell preparation can be labelled with several fluorochromes which all emit a different spectrum of light.

Prior to collecting data, the nozzle head was aligned to maximise the detection of fluorescent events. This was carried out using a mixture of two latex beads coated with fluorescein isothiocyanate (FITC) of different intensities. Control tubes were used to set compensation for dual labelled samples as phycoerythrin (R-PE) / propidium iodide (PI) signals interfere with the FITC signal and have to be adjusted for. Data analysis was performed using the PC-Lysis and CELLQuest® software packages (both from Becton Dickinson, Mountain View, CA).

2.9 Experiments to investigate expression of specific proteins

2.9.1 Dot blotting of total cell lysates

Cell lysates of known protein concentration were serially diluted in NP-40 lysis buffer with protease inhibitors (section 2.6.1) to produce solutions containing 100 μg - 100 pg of protein in 200 μl samples. Protran BA 85 cellulose acetate membrane (Schleicher and Schuell) was pre-wet in milli-Q water and soaked in PBS for 5-10 mins before use. The filtration manifold was assembled as follows: two sheets of filter paper (Whatman 3 MM) pre-wet in PBS were placed on the filter support plate of the filtration manifold. The cellulose acetate membrane was placed over the absorbent filter paper, and the sample-well plate placed on top and clamped firmly into place. A low pressure vacuum was applied to the manifold and individual wells were washed with 500 μl of NP-40 lysis buffer. The samples were applied to the wells, filtered through and washed thoroughly with lysis buffer (500 μl). The membrane was then removed from the manifold, air dried and stored at 4°C until immunodetection was performed using the ECL technique described in section 2.9.5.

2.9.2 Denaturing SDS-PAGE (Laemmli, 1970)

In SDS polyacrylamide gel electrophoresis (SDS-PAGE) polypeptide migration is governed by molecular weight. Sodium dodecylsulphate (SDS) is an anionic detergent which binds stoichiometrically to proteins by wrapping around the polypeptide backbone in the presence of a reducing agent. The resulting SDS-protein complex has a net negative charge proportionate to the length of the protein. Migration is affected by the molecular weight of the protein with equal charge per unit length.

Glass plates (8 x 5 cm) were cleaned and separated with 1.5 mm spacers. Two 7.5% separating gels (1.5 mm thick, 8 cm x 5 cm) were prepared using a monomer solution of 30.8% acrylamide 2.7%: bisacrylamide (v/v, NBL Gene Science Ltd) shown in the table below. The mixture was degassed under vacuum for 15 mins prior to addition of 0.04% (w/v) ammonium persulphate (APS) and 0.04% (v/v) TEMED (BDH Laboratory Supplies). The gel solution was mixed and quickly poured between the glass plates to a depth of 1 cm below the bottom of the sample wells. The gel solution was overlaid with water-saturated n-butanol (10% (v/v) milli-Q water in n-butanol) and left to polymerise for 2 hours.

Solution	7.5%	4%
Monomer solution	5 ml	0.88 ml
4 x 1.5 M Tris-Cl (pH 8.8)	5 ml	
4 x 0.5 M Tris-Cl (pH 6.8)		1.66 ml
10% (W/V) SDS	0.2 ml	66 µl
milli-Q water	9.7 ml	4.06 ml
10% w/v APS	100 µl	33.4 µl
TEMED	6.7 µl	3.3 µl

After polymerisation, the overlay was poured away and the gel surfaces rinsed with 1.5 M Tris-Cl (pH 8.8). Two gels were clipped vertically into the electrophoresis apparatus. A 4% stacking gel was then prepared as shown in the table. The solution was degassed, the polymerising reagents mixed and then quickly layered on top of the separating gels. A comb was inserted (10-wells x 1.5 mm) and the stacking gel left to polymerise for 1 hour. Meanwhile, cell lysates (usually 50 µg total protein per gel) were diluted in 2 x protein sample loading buffer (appendix I) upto total volume of 40 µl, boiled for 90 seconds in a water bath, then chilled on ice for 30 seconds to denature the proteins prior to electrophoresis.

Following polymerisation, the gels were placed in the Biorad Protean IIxi Cell® electrophoresis tank and submerged in tank buffer. The combs were removed slowly and the wells rinsed with electrode buffer. Samples were loaded into the wells, and the lid placed on the gel unit and connected to a power supply. High molecular weight pre-stained protein marker standards (10 µl per lane, BDH, Laboratory Supplies, Poole) were run concurrently with samples. The current was adjusted to 30 mA per 1.5 mm gel, and the samples were electrophoresed for 2 hours. When the dye front reached the bottom of the gel the power was switched off, the buffer was removed and the apparatus disassembled.

2.9.3 Coomassie blue stain

A PAGE gel was placed in Coomassie Blue staining solution (appendix I) for 4 hours. The staining solution was then replaced with destaining solution (appendix I) for 8 hours. Once destained, the gel was then placed between filter paper and saran wrap, and dried on a slab-gel dryer (Atto). The band intensity on stained gels was proportional to the amount of protein present.

2.9.4 Western blotting

Proteins were immobilised on Protran BA 85 cellulose acetate membranes (Schleicher and Schuell) using a semi-dry western blotting method. The 4% stacking gel was removed from the gel and the separating gel equilibrated in Towbin transfer buffer for 15 mins. The membrane and 6 pieces of filter paper were cut into rectangles of equal size as the gel, pre-wet with distilled water, then saturated in Towbin transfer buffer (appendix I). 3 pieces of saturated filter paper were stacked horizontally in the transfer apparatus. The membrane was placed on top, and then the gel. The remaining 3 sheets of filter paper were stacked on top. Air bubbles were removed from the stack by rolling with a pipette and the cathode was placed on top to seal the apparatus. Protein was transferred for 75 mins at 0.8 mA/cm^2. The stack was then dismantled, and the blot labelled and stained with Ponceau S solution (Sigma) for 5-30 mins. Excess dye was removed by rinsing in water and the molecular weight standard bands were marked with pencil. The blot was then fully destained in water and then blocked overnight (section 2.9.5).

2.9.5 Immunodetection by enhanced chemiluminescence

Non-specific binding sites on western blots were blocked by immersion in 5% (w/v) non-fat milk powder, 1% (w/v) goat serum and 1% (v/v) Tween-20 in PBS (Blotto) for one hour at 25°C on a rocking tray, or overnight at 4°C. The membrane was rinsed in two changes of wash buffer and then washed once for 15 mins, and twice for 5 mins with fresh changes of wash buffer. During the washing step the 1° antibody was diluted to 10 µg/ml unless otherwise stated, in 5 ml of 20% FCS in DMEM.

The membrane was incubated in 1° antibody solution for 1 hour at 25°C and washed as above. The horse radish peroxidase (HRP) conjugated 2° antibody was diluted to 1 μg/ml in 20% FCS/DMEM. The membrane was incubated in 2° antibody solution for 1 hour at 25°C and washed for 15 mins, and then a further 5 mins in fresh changes of wash buffer. Thorough washing was essential to reduce background activity.

An enhanced chemiluminescence kit (ECL, Amersham Life Science) was used to visualise immunolabelled peptides immobilised on western blots. An equal volume of detection solution 1 (luminol) was mixed 1:1 with detection solution 2 (p-iodophenol, both supplied in the kit) to a final volume of 0.125 ml/cm^2 membrane. The excess wash buffer was drained off the membrane and detection solution was poured onto the protein-bound side of the membrane. The membrane was incubated for precisely 1 minute at 25°C after which excess solution was drained off. The membrane was placed protein side up onto a piece of saran-wrap, taped into an autoradiography cartridge (Cronex Quanta Fast Detail Cassette, DuPont). The membrane was covered in saran-wrap, avoiding incorporation of air bubbles. In a darkroom autoradiographic film (Fuji, Medical X-ray Film) was placed on top of the membrane and exposed for 30 seconds and 5 mins. This film was developed and assessed. The exposure time of the next film was adjusted accordingly.

2.10 Preparation of cDNA from mRNA

2.10.1 Purification of mRNA from cultured cells

mRNA was prepared using the QuickPrep® Micro mRNA Purification kit (Pharmacia Biotech). This has been optimised for the isolation of mRNA from small numbers of eukaryotic cells in the presence of guanidium thiocyanate. 1x10^6 harvested cells were aliquotted into sterile universal tubes, washed in PBS and pelleted. Pellets were stored at -70°C until use. The mRNA extraction protocol was performed under 'RNase free' conditions.

The cell pellet was resuspended in 400 μl extraction buffer and homogenised by inverting for 2 mins at 25°C before diluting with 800 μl elution buffer. 1 ml of Oligo(dT)-cellulose bead slurry was placed in a sterile microcentrifuge tube. Concurrently, the cell lysate and Oligo(dT)-cellulose slurry were centrifuged for 1 minute at 10,000 x g. The storage buffer was aspirated from the Oligo(dT)-cellulose bead pellet. The cleared homogenate was placed on top of the Oligo(dT)-cellulose pellet and mixed by inversion for 3 mins. The slurry was then centrifuged for 10 seconds and the supernatant discarded. The Oligo(dT)-cellulose bead pellet containing bound poly-adenylated mRNA was then resuspended in 1 ml of high-salt buffer, and recovered by centrifugation for 10 seconds as before.

The beads were washed in this manner 5 times and then washed with 2 volumes of low-salt buffer. The pellets were resuspended in 0.3 ml of low-salt buffer and transferred to MicroSpin™ Columns placed in microcentrifuge tubes. The beads were spun onto the columns for 5 seconds and then washed with 3 x 0.5 ml of Low-salt buffer. mRNA was eluted from the column resin with two 200 µl volumes of Elution buffer pre-warmed to 65°C in a sterile microcentrifuge tube. mRNA in solution was concentrated by precipitation following the addition of 10 µl glycogen solution, 40 µl potassium acetate and 1 ml of 95% ethanol at -20°C. The mRNA was allowed to precipitate for 30 mins at -20°C and was then pelleted by centrifugation in a microcentrifuge tube 10,000 x g for 5 mins at 4°C. The precipitate could be stored under ethanol at -70°C for up to one year.

To recover the mRNA, the precipitate was spun for 20 mins at 10,000 x g in a bench-top centrifuge (4°C, Biofuge Fresco, Heraeus Instruments). The resulting pellet was washed with 70% ethanol, repelleted, then dried briefly by inverting the tube on absorbent paper. The precipitated mRNA was redissolved in 10 µl DEPC-treated water and the tube flicked, pulse spun and then heated to 65°C for 2 mins to help resuspend the RNA. The suspension was chilled on ice for 5 mins before being used (section 2.10.2).

2.10.2 Reverse transcription of mRNA samples

Complementary DNA (cDNA) was prepared from mRNA template using the Reverse Transcription Polymerase Chain Reaction (RT-PCR). 5 µl of mRNA sample were placed into RNase-free thin walled 65 µl tubes. A master reaction solution was prepared by mixing the regents in the table below (final concentrations are in parentheses).

Solution	(µl)
DEPC-treated water	5
5 x RT Superscript ™ 1st Strand buffer	5
Pd(N)6 (50 U/µl)	5
dNTPs (10 mM)	2
DTT (0.1 mM)	2
Recombinant RNase inhibitor (2 U/ml)	0.5
Superscript ™ Reverse Transcriptase	0.5
mRNA sample	5
Total volume	25

The volumes shown in the table were multiplied by the number of samples being prepared. Control samples were prepared with 5 µl DEPC-treated water in one tube. A second solution was prepared excluding reverse transcriptase (RT) to test for the presence of genomic DNA in the mRNA extracts. The reaction solutions were mixed and pulse-spun to ensure homogeneity. The solutions were then aliquotted into reaction tubes to produce a final reaction volume of 25 µl, and mixed. The solutions were overlaid with 20 µl of mineral oil to prevent evaporation, transferred to a thermocycler (Program Temperature Control System PC-700, Astec) and incubated at 37°C for 90 mins.

2.10.3 Polymerase Chain Reaction

cDNA samples generated from mRNA isolates by reverse transcription were amplified using the Polymerase Chain Reaction® (PCR). 3 µl of template cDNA were added to individual 65 µl thin walled tubes. A master reaction solution was prepared by mixing the solutions in the table below according to the oligo-primer being used.

Solution	Primers		
	GAPDH, OC, PTH (µl)	ER (µl)	BSP (µl)
milli-Q water	15.4	16.4	12.4
10 x Thermophilic buffer	2.5	2.5	2.5
$MgCl_2$ (25 mM)	2.5	1.5	2.5
dNTPs (10 mM)	0.5	0.5	0.5
Random Hexamer Primers	0.5	0.5	2
Taq DNA Polymerase	0.1	0.1	0.1
cDNA sample	3	3	3
Total	25µl	25µl	25µl

The volumes were multiplied by the number of samples being prepared, and contained 20 pmol of each primer and 1U of Taq DNA polymerase (Promega). The reaction mix was vortexed, pulsed and quickly added to tubes containing cDNA to produce final reaction volumes of 25 µl. The samples were vortexed, and then overlaid with 20 µl of mineral oil.

The mixtures were placed into a thermocycler (Astec) and subjected to cycles of denaturation, annealing and extension as follows:

- **Glutaraldehyde 3-phosphate dehydrogenase (GAPDH)**: initial denaturation at 94°C for 4 mins, 30 cycles at 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, followed by a final extension at 72°C for 7 mins.
- **Osteocalcin**: conditions were the same as for GAPDH.
- **Bone sialoprotein**: initial denaturation at 94°C for 5 mins, 30 cycles at 94°C for 1 minute, 50°C for 1 minute and 72°C for 2 mins, followed by a final extension at 72°C for 3 mins.
- **Parathyroid hormone receptor**: initial denaturation at 95°C for 5 mins, 30 cycles at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute, followed by a final extension at 72°C for 3 mins.
- **Oestrogen receptor**: initial denaturation at 95°C for 5 mins, 32 cycles at 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, followed by a final extension at 72°C for 3 mins.
- **PDGF A chain:** initial denaturation at 94°C for 5 mins, 35 cycles at 94°C for 45 seconds, 60°C for 45 seconds and 72°C for 2 mins, followed by a final extension at 72°C for 7 mins.
- **PDGF B chain:** initial denaturation at 94°C for 5 mins and a 5 minute anneal at 60°C followed by 35 cycles of 1.5 mins at 72°C, 45 seconds at 94°C and 45 seconds at 60°C for 1 minute, followed by a final extension of 10 mins at 72°C.

To calculate the annealing temperature the following rule of thumb was used: 20-50mer with 50-60% GC content): temperature (°C) = 4 x (G + C) + 2 x (A + T) - 6. cDNA samples were stored at -20°C for up to one year.

2.10.4 Agarose submerged gel electrophoresis

Following the PCR reaction, the size of the cDNA products were analysed by agarose gel electrophoresis to determine their size. Agarose (Routine electrophoresis grade, Flowgen) was dissolved in 1 x Tris Acetate-EDTA buffer (TAE, 50 ml) by heating to 95°C, to make up a 1.8% (w/v) agarose gel. The gel was cooled to 50°C (hand hot) and ethidium bromide (0.05 µl/1 ml of 10 mg/ml stock) was added and mixed. The gel was poured into a sealed electrophoresis tray. A comb was inserted and the gel was then left to set at room temperature for a minimum of 20 mins. Once set the seal was removed from the tray, the gel immersed in 1 x TAE buffer in an electrophoresis tank and the comb removed.

5 x DNA loading dye (appendix I) was mixed with 5 µl of RT-PCR product. The samples were loaded onto the gel and electrophoresed, alongside marker DNA (400 ng φx174-RF Hae III digest, Pharmacia Biotech) for 30 mins at a constant voltage of 100V. DNA migrated towards the anode and was visualised by illuminating the gel with UV light on a transilluminator (λ = 302 nm, UVP).

2.10.5 Southern blotting (Southern, 1975)

Sequence specific probe hybridisation was used to verify the cDNA product of RT-PCR. DNA was immobilised onto a nylon membrane by Southern blotting so that a fragment of ^{32}P-labelled DNA complementary to a sequence of interest could bind to it.

Following separation of DNA fragments by agarose gel electrophoresis (section 2.10.4) and examination of the gel by UV analysis, double stranded DNA was denatured by placing the agarose gel into two volumes of 500 ml of 0.5 M NaOH at 25°C, with periodic agitation each for 30 mins. The Southern blot apparatus was set up as follows (refer to figure 2.1): a glass plate was placed on an inverted support inside a tank. A filter paper wick (Whatman 3 MM) was cut to the same width as the gel, longer than the support, soaked in fresh 0.4 M NaOH and placed over the plate with the ends hanging into the tank as a wick. Onto this was placed 2 sheets of filter paper cut to the same dimensions as the gel. 500 ml of 0.4 M NaOH were poured into the tank to cover the ends of the wick and moisten the paper. The filter was smoothed onto the plate by rolling with a pipette to remove trapped bubbles. Parafilm was used to seal the edges of the paper. The gel was removed from the denaturing solution, flipped over so the lanes faced down, and aligned on top of the wick. The gel was smoothed down to remove trapped bubbles. A pre-cut nitrocellulose filter (NC, Hybond N+, Amersham Life Sciences), labelled with the date and experiment was placed face down onto the gel and smoothed. 2-3 more pieces of filter paper were placed on the NC. 6 sheets of flat, dry paper towels were placed over the filters and additional 3 cm thick stacks of paper towels were placed on top. A glass plate and a weight (250 g) were placed on top of the stack.

DNA was allowed to transfer for 5-16 hours before disassembly. The weight, top glass plate and paper towels were removed and the protruding wick ends trimmed. The filters/NC/gel 'sandwich' was inverted and the wick removed (now on top). Pencil was used to mark sample well positions on the NC. The gel was discarded and the membrane placed in 2 x SSC for 5 mins to remove NaOH. The membrane was allowed to dry briefly and then sealed in a plastic bag and stored at -20°C until probed.

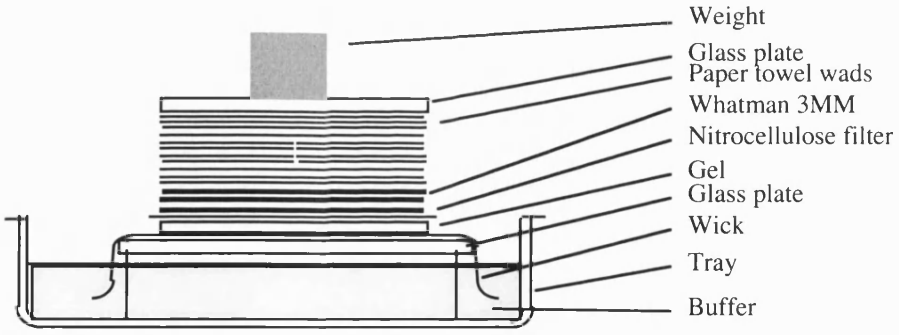


Figure 2.1 Schematic diagram of final layered organisation of materials

2.10.6 Probing southern blots with 5'-end labelled oligomers

A complementary 20mer oligonucleotide was labelled for use as a probe. Southern blotted NC filters were placed in a hybridisation bottle and blocked in 20 ml prehybridisation solution for 2 hours at 65°C, except the BSP probe for which prehybridisation and hybridisation were carried out at 55°C. During prehybridisation the probe was radiolabelled. The following solutions were placed in a microcentrifuge tube, forming a total volume of 10 µl, mixed, spun and incubated at 37°C for 30 mins, then 65°C for 20 mins.

Solution	µl
50 pmol oligo	1
10 x PNK buffer	1
milli-Q water	6
T4 Polynucleotide Kinase (10U/µl)	1
γ-^{32}P ATP	1

The labelled probe was separated from unincorporated label using a Nick column (Sephadex G-50 fine DNA grade, Pharmacia Biotech). The column was inverted several times to resuspend the gel, and allowed to settle upright held in a retort stand and clamp. The caps were removed and the column drained, and tapped to remove air bubbles. 1 ml equilibrium buffer (6 x SSC) was added to the column and drained. The column was the placed in a centrifuge tube and 2 ml of 6 x SSC were added and the column drained. The column was centrifuged for 4 mins at 514 x g in a centrifuge with a swinging-bucket rotor. The eluate was discarded. The column was then placed over a 1.5 ml microcentrifuge tube in the tubes provided in the kit, and the radiolabelled sample applied to the surface of the gel. The sample was eluted by centrifugation at 514 x g for 4 mins. The microcentrifuge tube containing the radiolabelled probe was removed.

After prehybridisation the solution was removed from the blots. These were rinsed in hybridisation buffer then replaced into the hybridisation bottles. 5 ml of hybridisation buffer (appendix I) was added to the bottles together with the probe solution. Fixed DNA was hybridised overnight at 65°C (55°C for BSP) in a rotating hybridisation oven (Hybaid). The following day the NC membranes were washed in 10 ml of each of the following solutions at 65°C (55°C for BSP) for 2 mins:

Washes	Solution
2	2 x SSC + 0.5% (v/v) SDS
2	0.1 x SSC + 0.5% (v/v) SDS
1	0.1 x SSC

Non-specific hybridisation was monitored with a hand-held β-counter (series 900 mini-monitor, Mini-Instruments Ltd). This was removed with repeated treatments of wash buffer. When background radioactivity was depleted, excess liquid was drained the from membrane, which was sealed in plastic. The filter was placed against autoradiographic film (Fuji, Medical X-ray Film) in an autoradiography cassette (Cronex Quanta Fast Detail Cassette, DuPont) and screened at -70°C initially for 2 hours. This film was developed and assessed. The exposure time of the next film was adjusted accordingly.

2.10.7 Image analysis

In order to gauge a change in the level of expression of peptides, DNA or mRNA, the relative amount of product on a gel or blot was quantified by densitometry. The gel or blot was placed on a UV transilluminator (UVP) or light box (Fostec) and the image was captured using a camera (TM-765 Kinetic Pulnix with cosmicar TV zoom lens) and a computer package (Imagedok, Kinetic Imaging Ltd.). Optical densities of bands were analysed with using a second computer program (Phoretix, Kinetic Imaging Ltd.). After manually selecting the bands, the intensity of each band was calculated and expressed as an arbitrary number presented as a table of results.

In experiments measuring levels of mRNA, GAPDH was used as a housekeeping gene. The intensity of the signal obtained following blotting and probing of GAPDH RT-PCR products reflects the quantity of total RNA and should be equal in all lanes if the same number of cells were used, or if the same amount of template was introduced to the reaction. Any variation in the GAPDH band intensities was removed by correcting the amount of cDNA in RT-PCR preparations. Optical densities were calculated as treatment over control ratios. At best this method gives semi-quantitative results.

2.11 Transfection methods

2.11.1 Culture of Bacteria Harbouring Plasmid DNA

Plasmid vectors incorporating genes with immortalisation properties were harboured in DH5α (*E. coli,* RecA$^-$ strain) glycerol stocks, stored at -70°C. DH5α were defrosted and suspended in sterile Luria-Bertani (LB) broth. This was prepared by adding 1% (w/v) Bactotryptone (Oxoid), 0.5% (w/v) yeast extract (Oxoid) and 1% (w/v) NaCl to milli-Q filtered water and adjusting the pH to pH 7.5 with 1 M NaOH. The broth was sterilised by autoclaving (123°C, 16" Hg, 20 mins). Ampicillin was added to the media (25 μg/ml sodium ampicillin) to select *E. coli* harbouring plasmid. The inoculated broth was incubated at 37°C for 3 hours in an orbital shaking incubator (15 x g, New Brunswick Scientific).

LB agar was made up as the broth, but with the addition of agar (15% w/v) following adjustment of the pH. LB agar (15 ml) was poured into 85 mm petri-dishes and stored inverted at 4°C until use. Bacteria were plated onto agar using a sterile loop and left to incubate overnight at 37°C. LB broth (10 ml) was inoculated with a single large colony which had grown on the agar and the culture was incubated overnight (37°C). The culture was then made up to 1 litre with fresh broth and antibiotic, and the mixture incubated at 37°C with constant agitation to produce large bacterial growth.

2.11.2 Purification of DNA from Bacterial Cultures

Plasmid DNA was isolated from overnight LB-broth cultures using the Wizard™ Maxipreps DNA Purification system (Promega). Cultured bacteria were pelleted by centrifugation at 10,000 x g for 15 mins and resuspended in Cell Resuspension solution. Cell Lysis solution was added for 2 mins and then mixed by inversion with Neutralisation solution. The mixture was centrifuged (10,000 x g, 15 mins) and the clear supernatant decanted into a clean centrifuge bottle. 0.6 volumes of isopropanol were added to each sample, and after thorough mixing, the samples were cooled on ice for 30 mins to precipitate the DNA. DNA was recovered by centrifugation (10,000 x g, 15 mins) and the pellet resuspended in Tris-EDTA (TE) buffer.

To purify plasmid DNA from the pelleted material, Purification Resin was mixed with the resuspended material by swirling. The column apparatus was arranged and the plasmid DNA / resin mixture poured into the column. Column Wash solution was applied to rinse resin out of the column, and the column was then spin dried. Preheated TE buffer (65°C) was applied to the column, left for 1 minute, and centrifuged at 10,000 x g to elute plasmid DNA. This DNA was aliquotted and stored at -20°C.

The composition of solutions used for the Promega Magic™ Maxipreps Purification system is shown in appendix I. Wizard™ Maxipreps DNA Purification system provides ≥ 500 µg DNA/sample or Qiagen Plasmid Purification Protocol (upto 10 mg DNA/sample). The Qiagen protocol employed similar methodology as described in this protocol.

2.11.3 Spectrophotometric analysis of DNA concentration.

The concentration of DNA solutions were determined spectrophotometrically in a LKB Biochrom Ultraspec II. Absorbances were blanked against the buffer of the DNA suspension. A sample aliquot was diluted in 250 µl and placed in a quartz cuvette (LKB, 500 µl 'H' cuvette). The absorbance of DNA was measured at 260 nm and protein contaminants at 280 nm. The concentration of DNA was calculated from A_{260} spectrophotometric absorbance values using the equation below where the DNA factor is 50 µg/ml of DNA when the optical density (OD) is 1 at 260 nm.

$$[\text{DNA}]\ (\text{mg}/\text{ml}) = A260 \times \text{DNA factor} \times \frac{\text{Volume of Cuvette (ml)}}{1000\ (\text{ml})} \times \frac{\text{Total sample volume (ml)}}{\text{sample volume (ml)}}$$

The absorbance at 280 nm was equivalent to the protein concentration in the sample, a ratio of 1.8 represents a pure DNA sample.

2.11.4 Phenol Chloroform extraction and Ethanol Precipitation of DNA

A volume of DNA solution was mixed with an equal volume of phenol (equilibrated with 0.1% (w/v) hydroxyquinoline and 0.2% (v/v) ß-mercaptoethanol) to form an emulsion. The mixture was centrifuged for 15 seconds at 10,000 x g (25°C) to separate the organic and aqueous phases. The upper aqueous phase was transferred to a fresh microcentrifuge tube. An equal volume of 1:1 phenol:chloroform was then added to the aqueous phase, mixed and centrifuged as before. An equal volume of chloroform was then added, mixed, centrifuged and transferred.

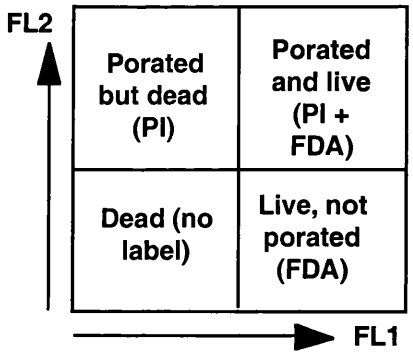
Salt was removed from the solution by ethanol precipitation. DNA was precipitated from the aqueous solution with the addition of 0.4 volumes of 5 M ammonium acetate. Two volumes of 100% ethanol were added to suspended DNA, the solutions were mixed by inversion and cooled to -20°C for 15 mins. The precipitate was centrifuged at 10,000 x g for 15 mins. The resulting pellet was washed with 70% ethanol, respun and dried briefly by inverting the tube on absorbent paper. The DNA pellet was resuspended in a specific volume of milli-Q water to produce a final DNA concentration of 1 µg/µl. DNA was also sterilised prior to transfection using this method.

2.11.5 Electroporation of adherent cells

CHO and MG-63 cells were cultured to 70% confluence, trypsinised and counted on a Coulter Counter (section 2.3.1). The cells were pelleted at 514 x g for 10 mins at 4°C. The supernatant was aspirated and the cell pellet washed with cold HBS (4°C). The cells were resuspended in HBS at a cell density of $1x10^7$ cells per ml. Plasmid DNA (10 µg/ml) was added to the cell suspension and the mixture left on ice for 5 mins. 500 µl of cell/DNA suspension was transferred to a pre-cooled electroporation cuvette (0.4 cm path length) and the cells were electroporated in a Biorad Gene Pulser. Following electroporation the cuvettes were left on ice for 15 mins. The cells were resuspended in DMEM supplemented with 10% FCS, replated into dishes and left to recover at 37°C, 5% CO_2 in air. After 48 hours the medium was aspirated and adherent cells were washed with 5 ml PBS. The medium was replaced with 10% FCS in DMEM, containing G418-S (50 µg/ml), $ZnCl_2$ (200 µM) and $CdCl_2$ (1 µM). The cells were harvested 24-48 hours later and transfection was investigated through detection of SV40 large T antigen expression by immunocytostaining, western blotting and β-galactosidase staining.

2.11.6 FACS determination of cell viability of electroporated cells

100 µl of electroporated cell suspension were removed and mixed with propidium iodide (PI, 10 µl of 100 µg/ml). The suspension was placed on ice for 10 mins, then diluted into 1 ml of 10% FCS in DMEM. Following a further 30 mins incubation on ice, the cells were pelleted (514 x g, 5 mins), washed in PBS and resuspended in 1 ml PBS. Fluorescein diacetate (FDA) was added (0.1 ng/ml) and the suspension incubated at 25°C for 10 mins. The labelled cell suspension was placed on ice during inspection by FACS. Cells were analysed by FACS as described in section 2.8.5. Detection of labelled cells were displayed on the FACS in the distribution as below:



2.11.7 *In situ* β-galactosidase staining

Cells expressing β-galactosidase following successful transfection with the pCH110 (Pharmacia Biotech, #27-4508-01, used at 50 μg/ml) reporter plasmid were stained with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-galactosidase (X-Gal, MacGregor *et al*, 1991). This plasmid contains the X-gal gene which encodes β-galactosidase. This cleaves 5-bromo-4-chloro-3-indolyl-β-galactose to form a coloured product staining transfected cells blue. Medium was aspirated from the transfected cell monolayer which was rinsed with 2 x 5 ml aliquots of PBS (pH 7.3). The monolayer was overlaid with 7.5 ml of fixative solution and incubated at 4°C for 5 mins. The fixative was aspirated and the cells rinsed again. The cell monolayer was overlaid with X-Gal solution and incubated at 37°C overnight. The solution was removed and the cells rinsed with PBS. The monolayer was then examined for positively stained blue cells using a light microscope (Wilovert, Wetzlar).

Chapter 3

Effect of culture conditions on CFU-F colony formation

3.1 Introduction

Colony forming units fibroblastic (CFU-F) comprise a subset of marrow stromal cells that are clonogenic and show a wide variation in their potential for proliferation and differentiation. Upon explantation of marrow stromal cells, CFU-F rapidly adhere to the culture substratum and proliferate to form colonies. The *ex-vivo* expansion and reimplantation of marrow stromal cells has been proposed as a treatment for a number of acquired and inherited diseases of the skeleton, and as an adjuvant in tissue reconstruction and repair following injury or surgery. For these therapies to be feasible large numbers of viable cells must be obtained in the shortest time possible to minimise the risk of infection, and the interval between their harvesting and reimplantation or grafting. The rate of expansion of these cells in culture is dependent upon the recruitment of CFU-F to the substratum, and subsequent proliferation of the progeny. Thus the aim of the work in this chapter was to optimise the growth of CFU-F in culture.

In order to optimise colony formation and expand the osteoblastic cell population *in vitro*, two elementary approaches were investigated. Initially, the influence of supplementing culture medium with ascorbate, and the synthetic glucocorticoid, dexamethasone, was ascertained. Secondly, the effect of altering the criteria under which human bone marrow stromal cells (BMSC) were explanted and cultured was investigated.

L-ascorbic acid (or vitamin C) is an essential factor required to sustain eukaryotic life. A deficit in bodily intake results in the condition of scurvy, with tenderness and swelling of joints, loosening of teeth, and anaemia (Levine, 1986). It is important in the maintenance of the integrity of mesenchymal-derived tissues including bone. Multifactorial activities of L-ascorbic acid are reported in many biological systems. In addition to its role as an antioxidant, L-ascorbic acid acts as a reductant for the iron prosthetic group of hydroxylase enzymes involved in biosynthesis of collagen, an important constituent of the extracellular matrix (ECM). A deficiency of vitamin C inhibits procollagen formation, resulting in degradation of connective tissues (Hata and Senoo, 1989). L-ascorbic acid (ASC) is very unstable in aqueous solution, at neutral pH, having a half-life of 2-3 hours at 37°C in culture medium (Peterkofsky, 1972). It is therefore technically difficult to maintain its concentration in culture. Treatment with a fluctuating concentration can be achieved with freshly prepared and filtered L-ascorbate. Alternatively, L-ascorbate 2-phosphate (ASP, Nomura *et al*, 1969) is very stable in aqueous solution, its activity being retained for 1 week when the derivative was dissolved in culture medium at 37°C (Hata and Senoo, 1989). A fixed concentration of ASP can be achieved in culture medium when the cells are fed twice weekly.

Numerous investigations have shown that bone mass is subnormal in patients with raised physiological levels of circulating glucocorticoids (Cushings syndrome) or in patients treated with steroids for conditions such as asthma and rheumatoid arthritis (Reid *et al*, 1986, Adinoff and Hollister, 1983). *In vivo,* glucocorticoids stimulate bone resorption and inhibit bone formation leading to an overall deficit in skeletal tissue (Delany *et al*, 1994). Glucocorticoid-induced osteoporosis primarily involves decreased width of trabecular bone (Adinoff and Hollister, 1983). The decrease in bone formation is thought to occur through a direct inhibition of osteoblastic function *in vivo* (Reid *et al*, 1986). Paradoxically however, at physiological concentrations *in vitro,* glucocorticoids have been shown to promote osteogenic differentiation in primary marrow stromal cultures from a number of species and induce formation of mineralised nodules in rat marrow stromal cell cultures (Owen *et al*, 1987, Maniatopoulos *et al*, 1988, Aubin *et al,* 1990, Bellows *et al*, 1990, Leboy *et al*, 1991, Davis *et al*, 1993, Beresford *et al,* 1994, Croisille *et al*, 1994, Cheng *et al*, 1994, Locklin *et al*, 1995). Culture in the continuous presence of a glucocorticoid has also been demonstrated to be essential for human marrow stromal cells to form mineralised tissue resembling bone when implanted into diffusion chambers *in vivo* (Gundle *et al*, 1995). Dx acts through glucocorticoid receptors, and exerts its effects on bone metabolism through modifications of gene transcription (Shalhoub *et al*, 1992).

A survey of the literature reveals a lack of consistency in the methods of BMSC culture. In this investigation an attempt was made to optimise BMSC seeding density and to increase the number of discrete colonies formed, as a linear correlation between colony formation and the number of mononuclear cells seeded had been reported (Bellows and Aubin, 1989, Owen *et al*, 1987). In preliminary experiments, it had been observed that removal of non-adherent cells from the adherent stromal cell layer after BMSC explant, increased colony formation. Since the development of the long-term bone marrow culture by Dexter and Lajtha (1974), there has been interest in the ability of the adherent cell layer of stromal cells to support haematopoietic cell growth. It is known that these two populations interact, and it is possible that the haematopoietic cells have an effect on the stromal cell population in culture (Herbertson and Aubin, 1995, Long *et al*, 1995, Castro-Malaspina *et al*, 1980, Castro-Malaspina *et al*, 1981, Friedenstein *et al*, 1992, Rickard *et al*, 1995). As it was not clear whether leaving these cells in culture was beneficial to the cultures, the effect of non-adherent cells on adherent cell colony formation was also investigated.

In this study, BMSC were explanted into primary culture with the addition of ASC or ASP to compare the effects of continuous or intermittent treatment on CFU-F colony formation and proliferation *in vitro*. The osteogenic influence of Dx was also studied. The concentrations of ASC, ASP and Dx used in these experiments have previously been shown to be optimal for the proliferation and differentiation of human bone-derived cells (Gallagher *et al,* 1996). The functional end-points used were clonogenicity, measured as the number of

colonies in quadruplicate dishes after 18 days primary culture, and stromal cell proliferation, measured as both the mean area per colony and the number of cells harvested from primary culture after 28 days growth. The prevalence of osteogenic precursors was estimated by counting the number of alkaline phosphatase positive colonies and by determining the biochemical activity of alkaline phosphatase in culture lysates.

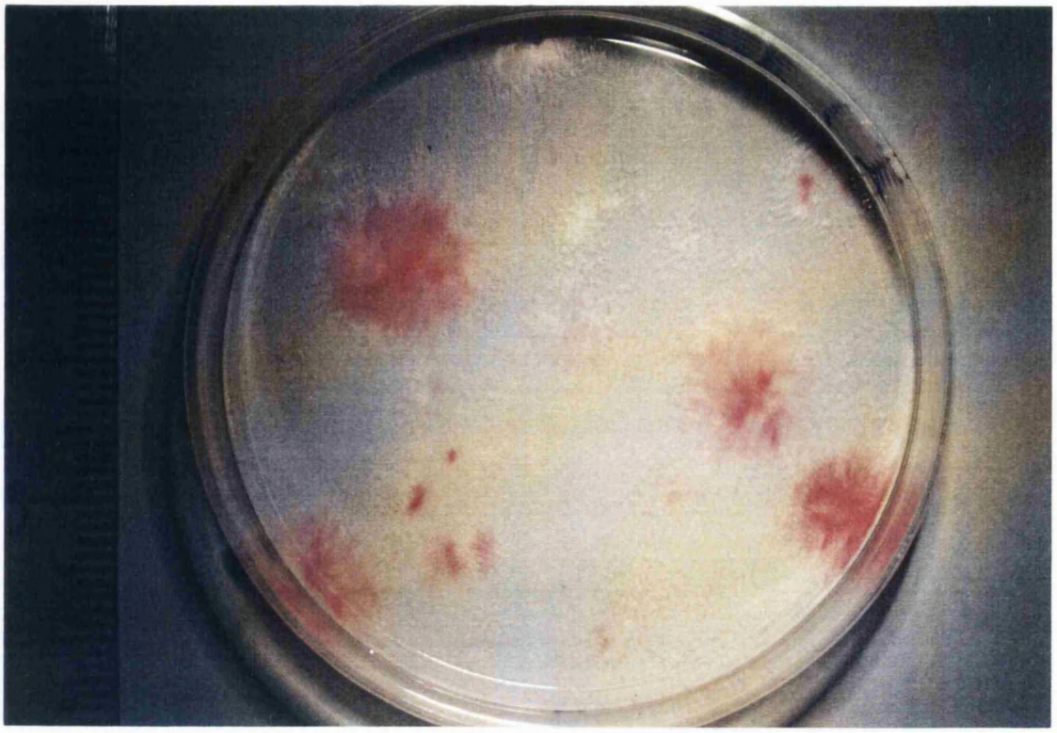
3.2 Results

3.2.1 Microscopic observations

Culture of bone marrow stromal cells under standard primary culture conditions resulted in the formation of colonies composed predominantly of fibroblast-like cells. Morphologically, these varied from long and spindle-shaped to flattened and multipolar with abundant stress fibres (figure 3.1). These overlapped in a parallel array to form a dense cellular meshwork. It was noted that although many cells initially adhered to the substratum post-seeding, only a proportion (~10%) subsequently spread and developed a spindle-shaped morphology. Many remained as single cells for the duration of culture. Proliferative cells first elongated, and then began dividing 4-24 hours post explant. Small colonies (20-100 cells) were visible by eye 4-7 days post-explantation. In each culture dish the colonies varied greatly in size (range 3-12 mm diameter), reflecting differences in cell morphology (flattened vs bipolar) and rates of proliferation. These differences were apparent within, and between donors. Colonies also varied in gross morphology. In some, cell density was uniform across the colony whereas in others it was greatest in the centre of the colony. In these latter colonies, the cells were often observed to radiate in a spiral array out from the centre of the colony. Between patient samples, colonies could either be discrete or overlapping, irrespective of the seeding density of the mononuclear cells or colony size.

The Fast-red TR dye method used for histochemical demonstration of alkaline phosphatase (AP) expression resulted in deposition of a red reaction product at sites of enzyme activity. Of 56 donors, 7 (12.5%) gave rise to colonies that all stained positive for AP, irrespective of culture conditions. In the remaining patient samples a proportion of colonies were AP positive, and this varied according to culture conditions and donor sample. Within AP positive colonies the distribution of AP positive cells was not uniform. Generally, they were localised in the centre of the colony where the cell density was greatest (figure 3.1ii). Based on the staining intensity, there was a wide variation in enzyme activity between AP positive colonies, both within and between donors.

i)



ii)

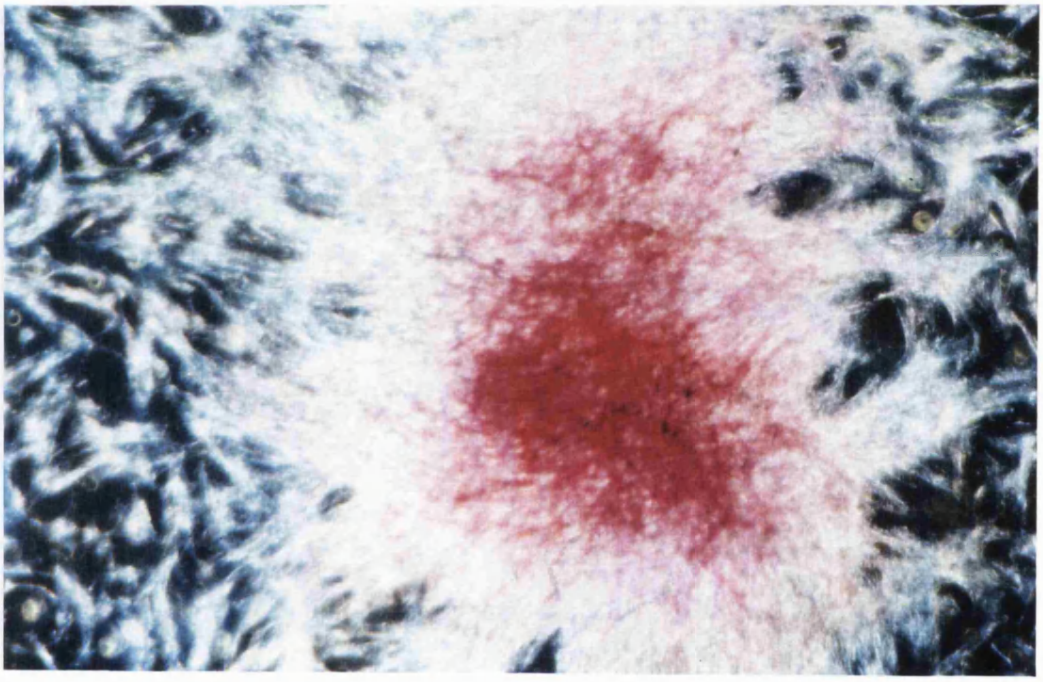


Figure 3.1 Examples of colonies derived from explanted BMSC

Mononuclear cells were isolated from bone marrow stromal tissue and cultured for 18 days under standard 1° conditions. Cells were then fixed with neutral buffered formalin (NBF) and stained with Fast-red TR. i) CFU-F colonies formed in a 55 mm diameter petri-dish (aside a mm scale, x 2 enlargement), ii) AP+ cells surrounded by AP negative cells of fibroblastic morphology in a CFU-F colony (40 x objective).

3.2.2 Comparison of effects of ascorbate on CFE and CFU-F proliferation

3.2.2.1 CFE

Compared with control, culture in the presence of continuous ASP did not affect total CFE, whereas in the presence of ASC it was reduced (figure 3.2). In contrast, both treatments increased the proportion of colonies expressing AP by ~100%.

3.2.2.2 Cell proliferation

In the presence of ASP, proliferation of CFU-F progeny was increased ~3 fold ($p < 0.05$, figure 3.3). In contrast, in the presence of ASC proliferation was unaffected.

3.2.3 The effects of Dx and ASP on CFU-F growth

3.2.3.1 CFE

Total CFE was unchanged in the presence of Dx or ASP and increased when both agents were added in combination (figure 3.4i). The observed changes were small in magnitude, but treatment with ASP and Dx attained statistical significance. AP positive CFE was increased in the presence of Dx and ASP. There was a further increase in AP positive CFE when both agents were added in combination. The proportion of total colonies expressing AP was increased in the presence of Dx or ASP, with ASP having a greater effect than Dx (figure 3.4ii). The effect of Dx and ASP in combination was additive.

3.2.3.2 Proliferation

3.2.3.2.1 Mean area per colony

Treatment with Dx had no effect on mean area per colony (figure 3.5). Irrespective of the absence or presence of Dx, the addition of ASP increased mean area per colony by ~20%.

3.2.3.2.2 Cell number

In the presence of Dx (figure 3.6) there was a small inhibition of cell proliferation (not-significant). In contrast, treatment with ASP produced a ~4 fold increase in cell number which was significant ($p < 0.05$). The effect of treatment with both agents in combination was not different to that of ASP alone.

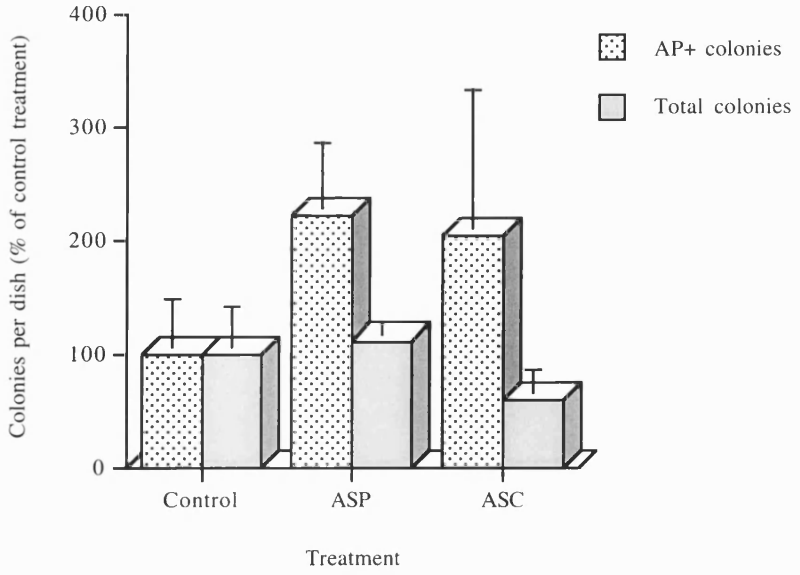
3.2.3.3 Alkaline phosphatase activity

Treatment with ASP or Dx alone (figure 3.7) increased the AP activity, 4 and 2.5 fold respectively. The effect of both agents in combination was slightly less than additive.

3.2.3.4 Mineralisation

Von Kossa positive deposits were observed only in cultures that had been treated with ASP and Dx in combination (figure 3.8).

i)



ii)

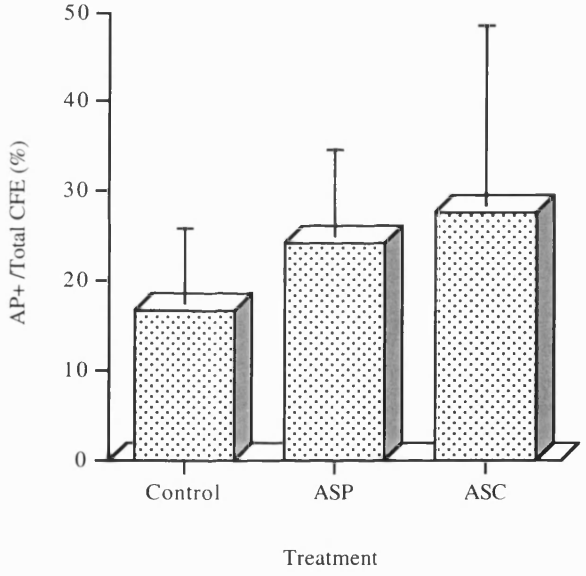


Figure 3.2 The effect of continuous and intermittent L-ascorbate treatment on CFE

BMSC were treated from explantation +/- L-ascorbate analogues, and cultured for 18 days. Colonies were fixed with NBF and stained for AP activity. Results show the mean of n = 3 donors +/- inter-donor SE. i) Percentage CFE of AP+ and total colonies compared to controls. The mean values for total and AP+ colonies in control cultures were 10.9 (+/- 1.2) and 1.2 (+/- 0.9) colonies per dish respectively. ii) AP+ colony formation as a percentage of total CFE under each treatment.

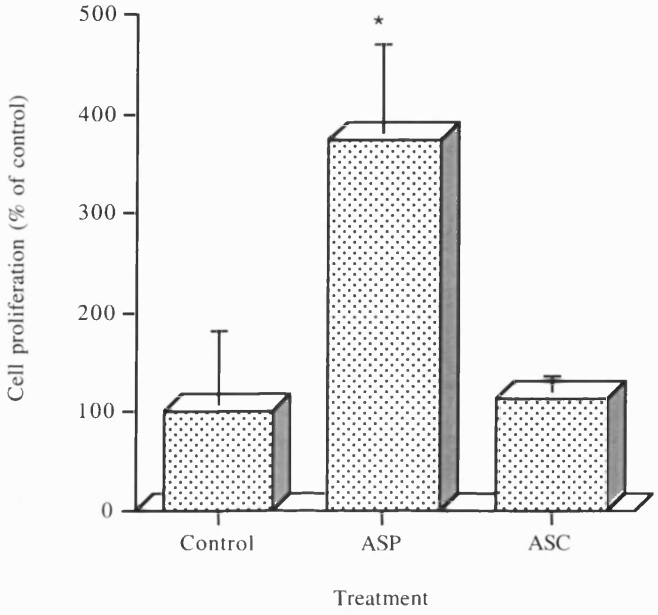
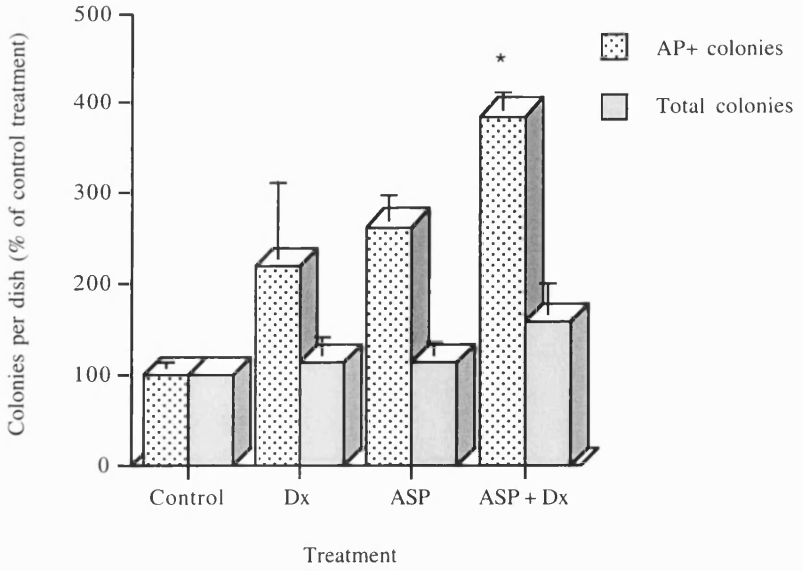


Figure 3.3 The effect of continuous and intermittent L-ascorbate treatment on proliferation

BMSC were explanted and cultured for 28 days +/- L-ascorbate analogues (10^{-4} M). Cells were harvested by treatment with sequential collagenase-trypsin and counted electronically. Results show the mean (n = 5) as percentage of control treatment +/- SE. * Indicates significantly greater proliferation than in control cultures ($p < 0.05$ using Kruskal Wallis Q test).

i)



ii)

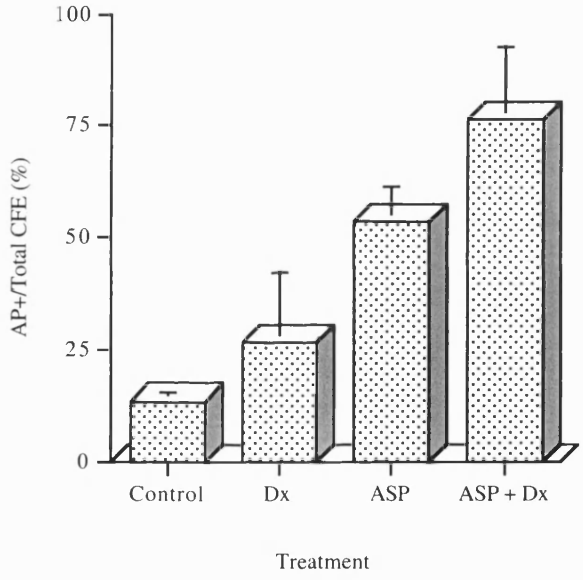


Figure 3.4 The effect of ASP and Dx on CFE

BMSC were cultured +/- 10^{-4} M ASP, and treated +/- 10^{-8} M Dx for 18 days. Colonies were then fixed with NBF and histochemically stained for AP expression. Results show mean of n = 7 +/- inter-donor SE. i) AP+ and total CFE as a percentage of control treatments (-/+ ASP) and ii) CFE of AP+ colonies as a proportion of the total colony formation. Where colony formation was 15.6 (+/- 1.6) and 13.6 (+/- 2.0) total colonies per dish, and 12.6 (+/- 3.1) and 7.2 (+/- 2.7) AP+ colonies per dish in control and ASP treated cultures respectively. * indicates $p < 0.05$.

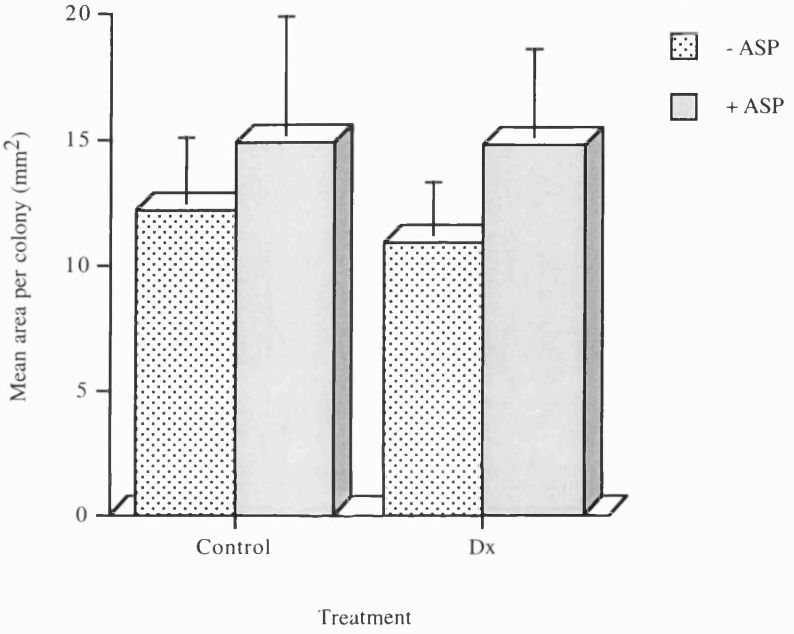


Figure 3.5 The effect of ASP and Dx on mean area per colony

BMSC were cultured +/- 10^{-4} M ASP, and treated +/- 10^{-8} M Dx. After 18 days 1° culture, colonies were fixed in NBF and stained with methylene blue. Results show the mean area per colony, (n = 4) +/- inter-donor SE. The colony diameter was measured (mm) using a ruler and the area of the colonies was calculated using πr^2, assuming the colonies were round.

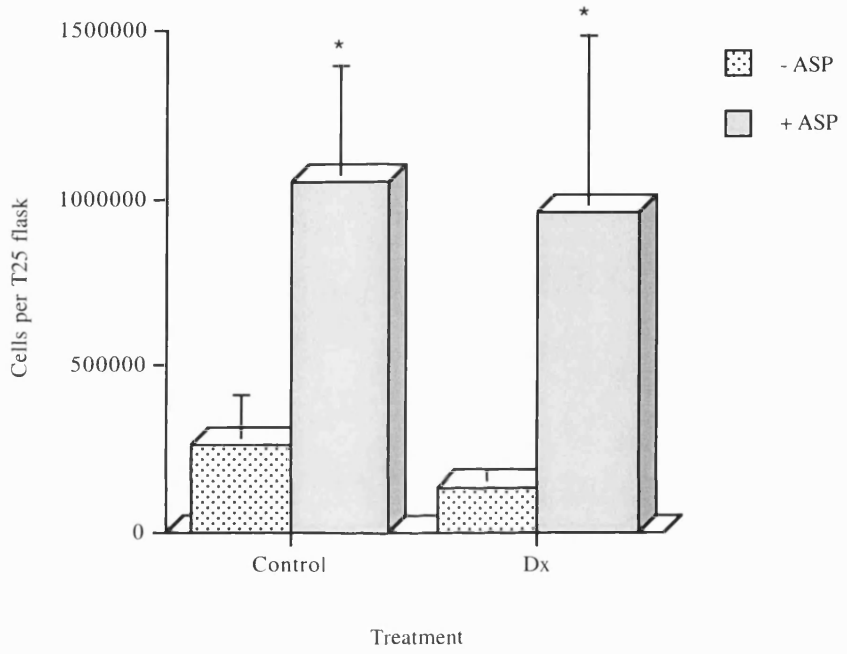


Figure 3.6 The effect of ASP and Dx on proliferation of CFU-F progeny

Bone marrow stromal cell 1° explants were cultured for 28 days in the absence or presence of 10^{-4} M ASP and 10^{-8} M Dx. Cells were harvested by treatment with sequential collagenase-trypsin and counted electronically. Results are the mean number of cells for n = 9 donors +/- inter-donor SE. * Indicates significantly greater proliferation than in control cultures +/- Dx ($p < 0.05$ using Kruskal Wallis Q test).

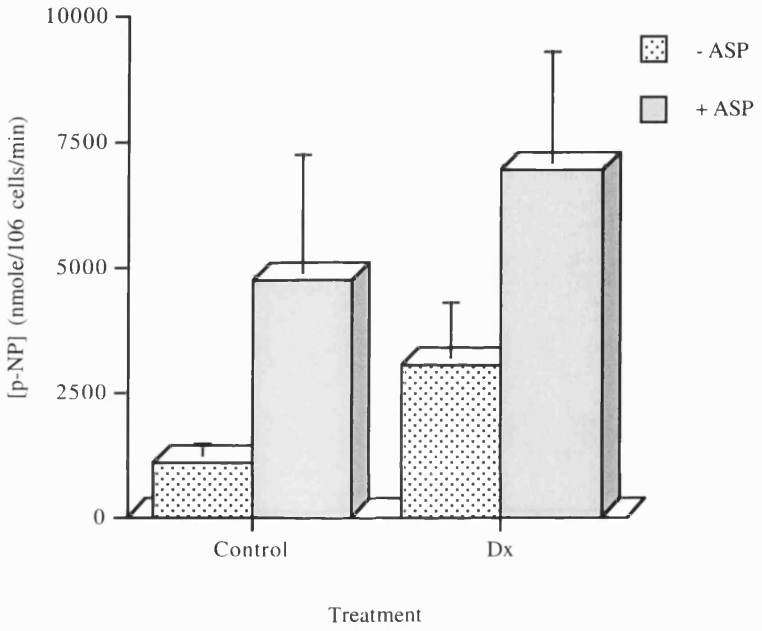


Figure 3.7 The effect of ASP and Dx on AP activity

BMSC were cultured under standard 1° conditions +/- 10^{-4} M ASP, and treated with 10^{-8} M Dx for 28 days. Cells were harvested by treatment with sequential collagenase-trypsin and AP activity was measured biochemically by p-NPP hydrolysis. Results show the mean (n = 4 donors) +/- SE.

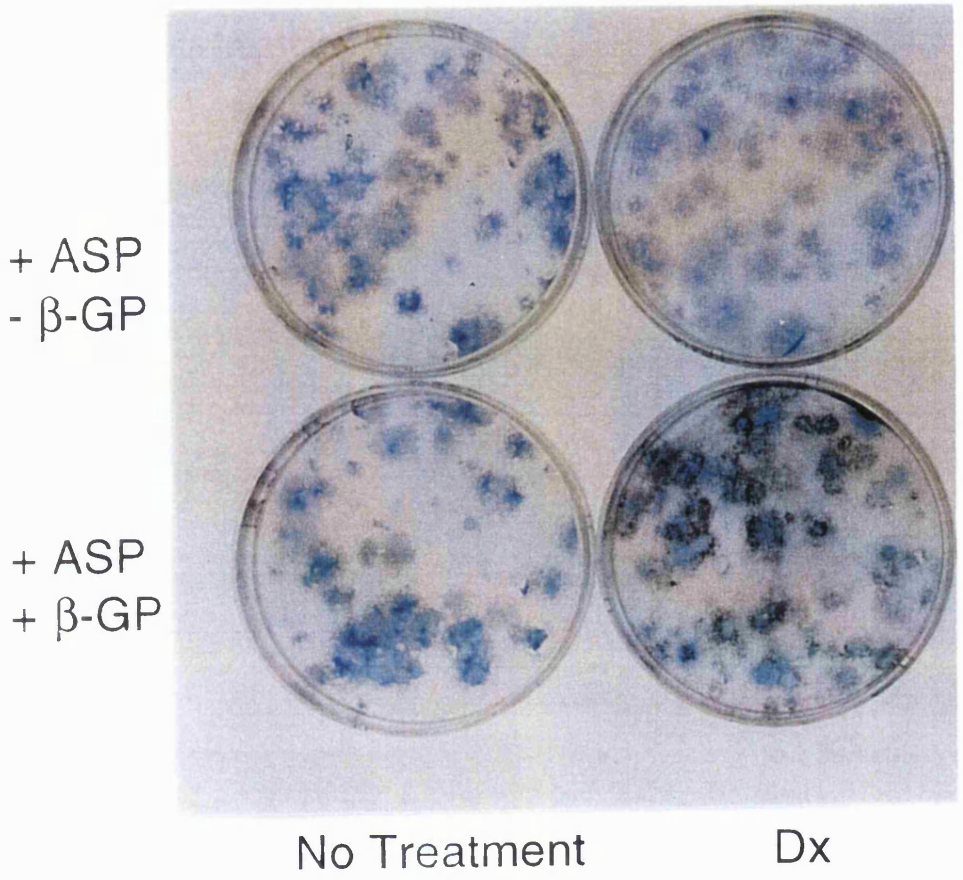


Figure 3.8 Mineralisation in cultures of BMSC with Dx treatment

BMSC were treated with 10^{-8} M Dx under standard culture conditions for 12 days. β-glycerophosphate was added to half the cultures for 5 days prior to their fixation with NBF. Colonies were stained with von Kossa stain and then counter stained with methylene blue.

3.2.4 The effect of varying the culture conditions on BMSC colony formation

3.2.4.1 Seeding density

Patient sample	Seeding density ($x10^4$ cells/cm^2)	AP+ colonies	Total colonies
#486	2	1.5 (+/- 0.4)	1.5 (+/- 0.4)
	4	17 (+/- 1.3)	17 (+/- 1.3)
	6	40 (+/- 2.8)	40 (+/- 2.8)
	8	87* (+/- 3.4)	87* (+/- 3.4)
#518	2	4.3 (+/- 0.5)	13.5 (+/- 1.7)
	4	6.3 (+/- 1.3)	21.5 (+/- 0.9)
	6	4.8 (+/- 1.6)	19.0 (+/- 4.4)
	8	6.8 (+/- 2.5)	28.8 (+/- 7.3)
#523	2	0.0	6.0 (+/- 0.7)
	4	1.1 (+/- 0.2)	8.0 (+/- 1.2)
	6	5.2 (+/- 2.1)	13 (+/- 3.7)
	8	6.0 (+/- 2.3)	23 (+/- 4.2)

Table 3.9 The effect of seeding density on CFE

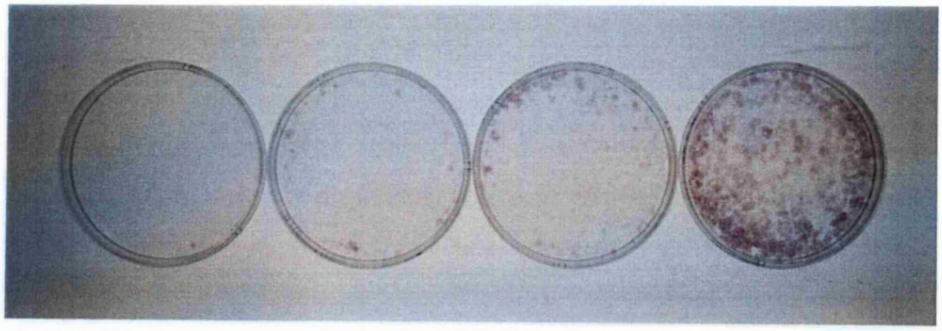
Results show mean (n = 4 petri-dishes) +/- SE for three donor samples.
* indicates a significant effect above $2x10^4$ cells/cm^2 ($p < 0.001$).

A positive association between total CFE and seeding density was observed in the 3 experiments (table 3.9, figure 3.10). In contrast, in 2 of 3 experiments the number of AP positive colonies formed did not increase with increasing cell density.

3.2.4.2 Time of removal of the non-adherent cells post-explantation

In preliminary experiments, it was observed empirically that CFE was reduced when non-adherent marrow cells were not removed at the first change of medium. On the basis of this observation, it was postulated that the population of non-adherent cells which remained suspended in culture medium influenced the clonogenicity of the adherent cells. To test this possibility, the influence of altering the timing of the removal of the non-adherent population on CFE was investigated.

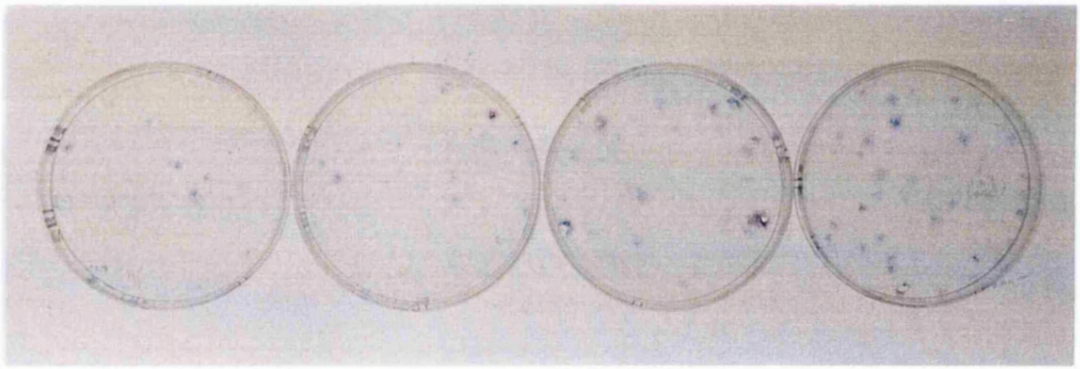
i)



2 *4* *6* *8*

Seeding density (cells $x10^4/cm^2$)

ii)



2 *4* *6* *8*

Seeding density (cells $x10^4/cm^2$)

Figure 3.10 The effect of seeding density on CFE

Two examples of an increase in colony formation with increased mononuclear cell seeding density (from donors i) #486 and ii) #523). BMSC explanted at increasing density were cultured for 18 days under standard conditions then fixed with NBF. Colonies were stained with Fast-red TR and counter stained with methylene blue.

Day of wash	Number of colonies in each donor sample				
	#388	#472	#481	#518	#523
1	14.7 (+/- 1.5)	12.7 (+/- 1.1)	6.50 (+/- 0,6)	13.3 (+/- 2.1)	5.80 (+/- 1.1)
2	16.3 (+/- 1.2)	20.8 (+/- 1.8)	5.50 (+/- 0,6)	0.00	1.00 (+/- 0.6)
4	11.0 (+/- 0.5)	30.3 (+/- 2.3)	12.3 (+/- 2.1)	0.00	1.70 (+/- 0.5)
7	7.00 (+/- 2.7)	17.0 (+/- 2.1)	ND	1.00 (+/- 0.4)	1.30 (+/- 0.5)
10	6.70 (+/- 1.2)	9.80 (+/- 1.2)	0.50 (+/- 0.3)	0.00	0.00

Table 3.11 The effect of removing non-adherent cells from 1° culture on CFE

BMSC were explanted and cultured under standard conditions in the presence of 10^{-4} M ASP and 10^{-8} M Dx. At the indicated time-intervals post-explantation, non-adherent cells and medium were aspirated from the culture dish. Adherent cells were washed in two volumes of PBS and thereafter fed twice weekly for 18 days. Colonies were fixed with NBF and total CFE were counted. The table shows the means (n = 4 petri-dishes) +/- SE. ND, not done.

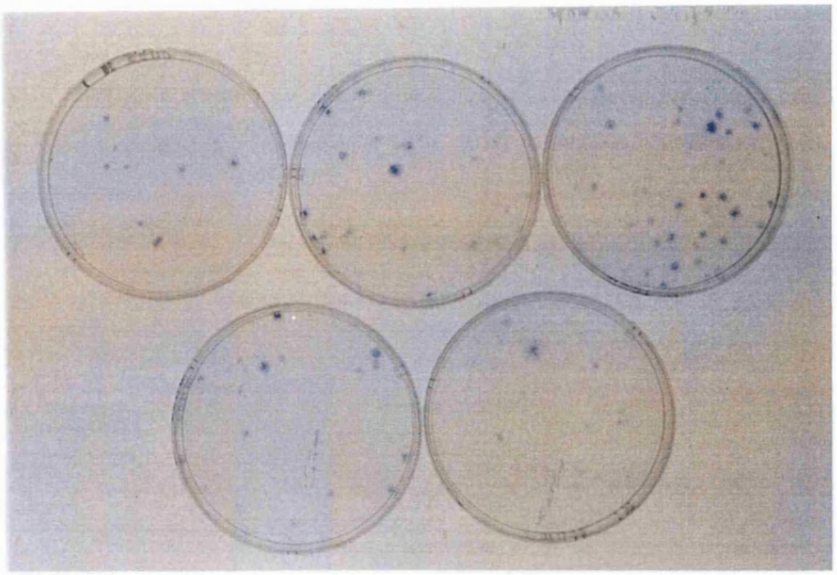
The results (table 3.11, figure 3.12) show that colony formation increased in 3 of 5 patient samples with time in culture, and peaked within 4 days. Continued co-culture of the adherent and non-adherent cell populations beyond 4 days was associated with a reduction in CFE.

3.2.5 Colony forming potential of the non-adherent population

Following the observation that non-adherent cells removed from primary BMSC culture at the first wash post-explantation formed colonies when replated into fresh petri-dishes, experiments were carried out to investigate the colony forming capacity of this population. Examples of the propensity of non-adherent cells to form colonies after replating are shown in figure 3.13. Colony formation occurred in 6 of 12 non-adherent cell cultures replated between 2 and 4 days primary culture. In 50% of these cases, however, only 2 or 3 colonies formed per 55 mm diameter dish, corresponding to a CFE of less than 10% of that seen in the original cell suspension. These colonies expressed AP. Colony formation decreased when the supernatant was replated with increasing time post-explanation and no colonies were formed when non-adherent cells were replated after 4 days of primary culture. Total colony formation was not affected by addition of ASP or Dx to cultures, although the number of AP positive colonies did increase with Dx treatment (figure 3.13i).

i)

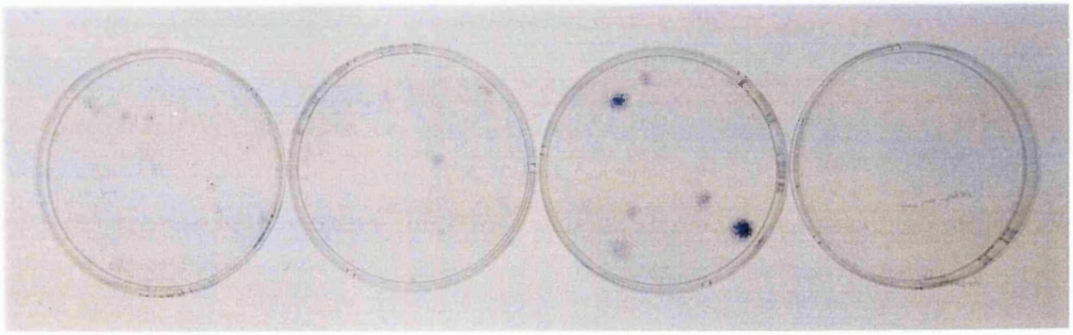
1 *2* *4*



7 *10*

Days post explant

ii)



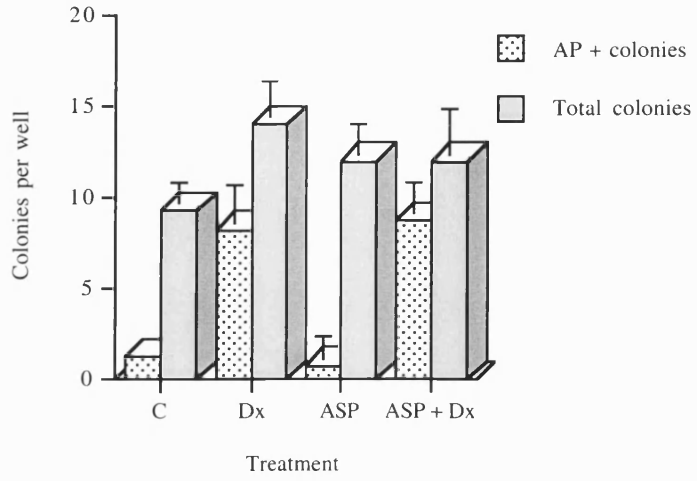
1 *2* *4* *10*

Days post explant

Figure 3.11 Removal of non-adherent BMSC from 1° culture affected CFE

BMSC were explanted and cultured under standard 1° conditions in the presence of 10^{-4} M ASP and 10^{-8} M Dx. At the indicated time intervals post-explantation, medium and non-adherent cells were aspirated, and adherent cells washed in two volumes of PBS. Thereafter, cultures were fed twice weekly for 18 days. Colonies were fixed with NBF, and counter stained with methylene blue.

i)



ii)

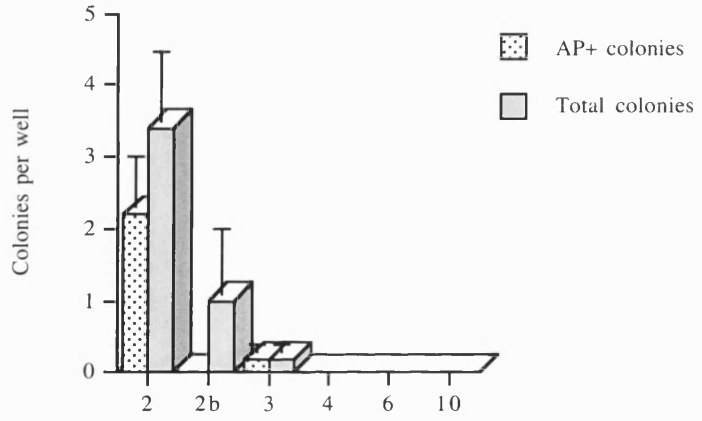


Figure 3.13 Colony formation by the non-adherent BMSC population

BMSC were cultured under 1° conditions, (i) +/- ASP and/or Dx and (ii) with ASP and Dx. On day 4 post-explantation (i), or intervals between days 2-10 (ii) the suspended non-adherent population and medium was removed from adherent cultures and replated into 6-well plates. Passaged non-adherent cells were left to adhere for 4 days, then washed in 2 volumes of PBS to remove remaining cells in suspension. In (ii) these non-adherent cells were repassaged in fresh 6-well plates (2b). Cells were then cultured under standard conditions for a further 14 days, then fixed in NBF and histochemically stained for AP. These results show the mean (n = 4 wells) +/- SE.

3.3 Discussion

Explantation of human marrow stromal tissue gave rise to colonies typically composed of cells of fibroblastic morphology. The first sign of these cells in culture was the appearance of single, isolated, fibroblast-like cells with a characteristic elongated morphology. Marrow cell suspensions prepared from different donors showed a wide variation in their propensity for colony formation. The observed variation was not obviously attributable to donor's age, sex, recent drug history or underlying pathology.

Depending on the influence of culture conditions and/or the level of commitment of the individual progenitor cells, colonies of different cellular phenotype formed. Variable rates of proliferation gave rise to colonies of different size in the same culture, resulting from differences in CFU-F phenotypes, and in their response to growth factors present in the serum supplement in culture medium. Most colonies contained AP positive cells, indicative of a degree of osteogenic differentiation, with AP negative cells located primarily around the colony perimeter, where proliferation is thought to be most rapid. The intensity of Fast-red TR stain varied between colonies, indicating that the cells expressed different levels of AP and were at different stages of maturation. It was apparent therefore, that the colonies formed in primary culture were derived from a heterogeneous population of adherent clonogenic cells, an observation in accordance with the findings of other investigators (Friedenstein *et al*, 1976, Owen, 1988, Kuznetsov *et al,* 1997a).

The effect of L-ascorbate supplementation was tested in primary bone marrow stromal cultures, and the use of ASP was found to be preferable to that of ASC. Benefits included an increase in cell number and an increase in the proportion of AP positive colonies. This is supported by results derived from a variety of tissues in which L-ascorbate alone promotes the proliferation and differentiation of BMSC *in vitro* (Leboy *et al*, 1989, Aronow *et al,* 1990, Ono *et al,* 1990, Archer *et al*, 1990, Owen *et al*, 1990, Gundle *et al*, 1995). The expression of AP induced by ASP treatment is undoubtedly a secondary effect of an increase in the synthesis and secretion of collagen type I. The presence of a well organised extracellular matrix provides a three dimensional structural support to which the cells adhere and modify their shape and behaviour, a permissive environment for cell proliferation and differentiation (Franceschi, 1992). It also acts as a binding site and reservoir for a large number of autocrine growth factors (Adams and Watt, 1993, Lin and Bissel, 1993, Sharpe and Ferguson, 1988). An increase in cell density and/or cell-cell contact is also thought to promote differentiation through stimulation of integrin mediated cytoskeletal pathways (Harada *et al*, 1991, Schubert, 1992). This may be responsible for the localisation of AP staining to the centre of colony where the cell density is greatest. Recently binding of OSF-2 to OSE-2 has been found to be upregulated in the presence of ASP (Xiao *et al,* 1997).

In the presence of ASP, addition of dexamethasone promoted colony formation. This may result from promotion of the adherence of a non-adherent cell population. Results also indicate that ASP and Dx promote stromal cell differentiation, and that their effects in this cell culture system are additive. Overall, the observed effects are consistent with the known beneficial effects of these agents on the proliferation and differentiation of cells of the osteoblast lineage (Grigoriadis *et al*, 1988, Beresford *et al,* 1994, Cheng *et al*, 1994, Gronthos *et al,* 1994, Krebsbach *et al*, 1997).

Although it has been widely found that long-term administration of glucocorticoids, or hypersecretion in pathological conditions, leads to profound osteopenia *in vivo*, paradoxically glucocorticoids are an important inducer of osteogenic differentiation *in vitro*. *In vivo* the effects of glucocorticoids are attributed to increased renal calcium excretion, and decreased intestinal absorption of calcium with a consequent increase in parathyroid hormone and direct actions of the hormone on osteoblasts and osteoclasts (Defranco *et al,* 1992). However, bone loss is considered to result largely from suppression of osteoblast function reflected by a reduced level of circulating osteocalcin (Reid *et al,* 1986a). *In vitro* Dx has been shown to increase total colony number and the number of colonies that express AP. However, the effects of Dx are markedly concentration and species specific. Treatment of marrow cultures with high Dx concentrations (10^{-5}-10^{-6} M) have been found to inhibit osteogenic progenitor growth and radiolabelled proline incorporation into collagen in primary rat cultures (Chen and Feldman, 1979, Scutt *et al,* 1996). The degree of dependence on glucocorticoids for osteogenic differentiation differs between species: in the rat it is absolute (Maniatopoulos *et al,* 1988, Tsuji *et al*, 1990, Leboy *et al*, 1991, Kasugai *et al*, 1991, Locklin *et al*, 1995), in man it is partial (Davis *et al*, 1993, Cheng *et al*, 1994, Beresford *et al*, 1994, Kuznetsov *et al,* 1997) and in the mouse it is inhibitory (Lian *et al*, 1997, Frenkel *et al*, 1997).

In these studies, treatment with Dx increased the proportion of AP positive colonies, but at an expense of decreasing cell proliferation. Dx treated cells became flattened, enlarged, and polygonal in long term cultures. The colonies in Dx treated cultures were generally smaller than in control cultures and contained more densely packed cells. Previous studies with rat and human bone-derived cell cultures demonstrated that Dx increased indices of osteogenic differentiation, at the expense of decreasing cell number (Herbertson and Aubin, 1995). Dx exerts its effects by modification in expression of osteoblast-related genes (Lian *et al*, 1997), including inhibition of collagen synthesis (Dietrich *et al,* 1979, Canalis, 1983, Shalhoub *et al*, 1992). In cultures of human bone-derived cells this is corrected in the presence of ASP when added in combination, therefore, these agents provide optimal conditions for the growth and differentiation of the AP positive cell population. Indirectly, Dx may also influence cell proliferation through modification of synthesis or activity of growth factors

and cytokines secreted by bone, for example expression of IGF-I and TGFß are directly down-regulated by glucocorticoid treatment (Chen *et al*, 1991, Centrella *et al*, 1991a).

In the few experiments in which the seeding density was investigated, a linear relationship was not evident between total or AP positive colony formation and the number of cells explanted. This is in contrast with the results of Bellows and Aubin (1989), who demonstrated a direct association between the number of cells plated and the number of nodules formed from cultured rat calvarial populations, and the results of Owen *et al* (1987) who showed a linear correlation between AP positive colonies and seeding density. However, these studies were carried out using rat marrow stromal cells and variation in results may be due to differences in preparation of marrow, culture conditions or species differences.

Interactions between cell types within these cultures has been observed in a number of studies. Aubin *et al* (1990) found that a linear relationship between seeding density and nodule formation was observed only when non-adherent cells were added into adherent rat marrow cell cultures, and that endothelial cell conditioned medium increased osteoprogenitor number. In co-cultures of rat fibroblasts and rat bone marrow stromal cells, Ogiso *et al* (1991) observed a reduction in nodule formation. These data and others (Hughes and McCulloch, 1991, Ogiso *et al,* 1991, Herbertson and Aubin, 1995) indicate that the growth of osteogenic cells may be under the regulation of other cells in the bone marrow which can exert either stimulatory or inhibitory activities. Notably the marrow stromal adherent population *in vitro* contains a great diversity of cell types in addition to cells of the osteoblast lineage, including fibroblastic cells, adipocytes (Aubin *et al*, 1990) and endothelial cells (Fei *et al,* 1990, Penn *et al,* 1993), and an increase in seeding density is also likely to increase the presence of other adherent cells in these cultures. In addition to marrow stromal cells, the adherent cell population includes macrophages (Castro-Malaspina *et al,* 1980, Song and Quesenberry, 1984, Herbertson and Aubin, 1992) and granulocytes (Simmons *et al*, 1991) depending on species. Although marrow stromal fibroblasts can stimulate the proliferation of these non-fibroblastic cell types there is no evidence of an influence of these cells over CFU-F colony formation. However, the juxtaposition of bone and immune tissue in the marrow stroma suggests that biochemical events between these cell components are likely to occur, and activated macrophages are capable of secreting many cytokines, which may interfere with stromal and osteoblastic cell growth. These include IL-1 and IL-6, which can effect bone resorption and formation (Rosen *et al*, 1990), and TNFα which directly inhibits collagen synthesis (Canalis, 1987, Bertolini *et al*, 1986).

Non-adherent BMSC have also been shown to both directly influence adherent stromal fibroblast proliferation (Castro-Malaspina *et al*, 1980, Castro-Malaspina *et al,* 1981, Hirata *et al*, 1985, Friedenstein, 1990) and mediate effects of other factors under investigation

(Rickard *et al*, 1995). Beyond four days primary culture, there was a tendency for the number of colonies to decline. This study provides evidence that prolonged co-culture of adherent marrow cells with non-adherent population can lead to a decrease in the number of fibroblastic colonies formed. It has previously been shown that proliferation of marrow stromal fibroblasts are affected by non-adherent cells in primary culture (Castro-Malaspina *et al*, 1980, Rickard *et al*, 1995). The non-adherent cells remaining in the primary cultures after four days were small in size, round, and viable based on the exclusion of trypan blue. Maniatopoulos *et al* (1988) noted the presence of small, round, mononuclear cells in their primary cultures, and attributed them to haematopoietic component of bone marrow. The cell cycle status of these cells was not investigated, but could be using Hoechst 33342 (Sigma) of which the staining intensity increases with cell DNA content. Haematopoetic cells may be explanted even following density gradient centrifugation (Keifer *et al*, 1991, Lymphoprep® assay protocol), and thus their numbers in culture may also be increased with an increase in cell seeding density. Stromal-haematopoietic non-contact cultures described by Hurley *et al* (1995) gave rise to more stromal colonies. Thus in our system removal of non-adherent cells with repeated washes of adherent cultures may limit interactions of haematopoietic and mesenchymal cells. Scutt and Bertram (1995) however, found that the timing of first medium change had no significant effect on CFU-F number, and Majors *et al* (1997) observed no effect on the number of AP positive colonies following removal of non-adherent cells after 24 hours in primary culture. In a recent publication, Kuznetsov and Robey (1997) studied the growth requirements for marrow stromal fibroblast colony formation from different species *in vitro*. In this investigation, the effect of feeder cells (irradiated bone marrow cells) on bone marrow cells growth in serum-containing medium was assessed. Mouse marrow stromal fibroblast precursors were feeder cell dependent, guinea pig were partially feeder cell dependent and human cells were independent, thus the effect of non-adherent cells in culture may also be species specific.

The results of this investigation demonstrate clearly that the formation of colonies continues up to four days post-explantation. CFU-F are highly adherent and it has previously been reported that ~95% will attach in less than six hours (Castro-Malaspina *et al*, 1980, Friedenstein *et al*, 1992). Surprisingly, recovery of the non-adherent cell population post four days revealed the presence of an additional population of cells with colony forming potential, when transplanted to a fresh culture dish. The presence of CFU-F in the non-adherent population has previously been shown in rat (Scutt and Bertram, 1995) and human (Oreffo *et al*, 1995) marrow stromal cultures. These results indicate the presence of two populations of potentially adherent cells in primary cultures of BMSC, one population which adhere soon after explantation (<6 hours), and a second which become adherent with time in culture, or with a change in culture conditions. This supports the suggestion that establishment of an adherent population results in the release of one or more factors that act to inhibit the further recruitment of cells from the non-adherent population (Castro-Malaspina

et al, 1980). The differences in adherence properties in these two populations may relate to their expression of adherence receptors such as cellular adhesion molecules, integrins, and basal lamina proteins such as laminin. The non-adherent cells are postulated to be at a more primitive stage in the mesenchymal lineage than adherent cells. This possibility is supported by the work of Long *et al* (1995), who isolated a population of non-adherent low density cells which contained two distinct cell populations, a population which formed clusters of mature osteoblastic cells, and a second which formed colonies of highly proliferative osteoblastic cells. It may also be true that marrow stromal tissue contains cells that are dislodged from bone surfaces which may represent a committed, adherent osteoblastic population, whereas the stromal tissue contains less mature cells within the fibrous matrix. Recently Rickard *et al* (1994) identified two bone progenitor cell populations when bone marrow stromal cells were stimulated *in vitro* with Dx and/or bone morphogenetic protein type 1 (BMP-1). One population differentiates into mature osteoblasts quickly, whereas a second retains its capacity for osteoblastic differentiation with further exposure to inducers.

The improvements obtained in colony formation and cell number through adapting the methodology in this study, whilst worthwhile, were of small magnitude relative to the variation noted between donor samples. These results impress that colony formation is essentially donor dependent. Although the number of cells seeded from each donor was constant, the colony forming efficiency was diverse within populations, indicating that the number of CFU-F per unit mononuclear cell count varies between patients, or that the colony forming efficiency of stromal cells from different individuals varies. A combination of these is probable. In two separate studies, Majors *et al* (1997) and Muschler *et al* (1997) reported a significant 70% variation in the number of CFU-F in marrow aspirates attributable to differences between donors; a 20% variation in CFU-F number between aspirates from the same donor was attributed to culture conditions. Much of the variation between reports of colony formation is likely to be due to differences in the site of bone marrow harvest, the harvesting and processing techniques, and the culture conditions used, including the concentration of dexamethasone. Lennon *et al* (1996), and Beresford *et al* (1993) have also shown that FCS is a critical factor in the culturing of marrow cells. Thus many factors are of importance when optimising conditions of colony growth. However, in further experiments, the seeding density of BMSC was maintained at $2x10^4$ cells/cm^2, and colony formation was enhanced by the addition of ASP to primary cultures. These were washed extensively at four days post-explantation to remove the effects of non-adherent cells. To expand the population of marrow-derived stromal cells other methodologies could be evaluated, and the use of cytokines and growth factors to increase colony formation and proliferation is worthy of consideration.

Chapter 4

An investigation of the effect of PGE_2 on CFU-F adherence

4.1 Introduction

Prostaglandin E_2 (PGE_2) is a locally active factor and one of the few agents known to stimulate bone formation *in vivo* (Kawaguchi *et al*, 1995). An anabolic effect of PGE_2 was first seen following its infusion into infants to prevent closure of the ductus arteriosis, which produced an increase of periosteal woven bone formation (Ueda *et al*, 1980). It has since been established that the administration of PGE_2 results in substantial increases in bone mass when applied systemically or locally in a variety of species (Jee *et al*, 1985, Jee *et al*, 1987, Norrdin and Shih, 1988). *In vitro*, PGE_2 has been found to stimulate the formation of mineralised nodules in rat calvarial cultures (Tang *et al*, 1996) and to increase total alkaline phosphatase activity, cell number, and collagen accumulation in rat marrow stromal cultures (Scutt and Bertram, 1995). Prostaglandins may also mediate the increase in bone formation that occurs in response to mechanical loading (Pead and Lanyon, 1989, Takagaki *et al*, 1996), growth factors, such as TGF-α and -β (Tashjian *et al,* 1985, Lerner, 1996), and systemic hormones such as PTH (Klein-Nulend *et al,* 1991). Prostaglandins are abundant in bone and are produced largely by cells of the osteoblast lineage (Kawaguchi *et al*, 1995). The two steps in prostaglandin production are the release of arachadonic acid from phospholipids, through the action of phospholipase A_2 (PLA_2) and the conversion of arachidonic acid by the bifunctional prostaglandin G/H synthase (PGHS) to PGE_2 (Smith, 1992). It is known that the effects of PGE_2 can be mediated by three different receptors termed EP_1, EP_2, and EP_3. The EP_1 receptor functions via the activation of PLA_2 followed by the release of diacylglycerol (DAG) and inositol triphosphate (IP_3) which in turn activate protein kinase C (PKC) and mobilise calcium, respectively. The EP_2 and EP_3 receptors function through an increase or decrease in cAMP synthesis respectively, followed by the activation or non-activation of protein kinase A (PKA, Scutt *et al*, 1995).

The cellular basis for the anabolic activation of PGE_2 is poorly understood. Woodiel *et al* (1996) postulated that prostaglandins have a dual mode of action, first stimulating the replication of osteoprogenitor cells and then promoting their maturation. In addition to their postulated direct effects, prostaglandins may also have indirect effects through the stimulation of insulin-like growth factor I (IGF-I) production, a widely expressed, abundant, autocrine and paracrine factor that regulates the proliferation and differentiation of a variety of cell types. PGE_2 potently stimulates IGF-I synthesis in bone through a cAMP pathway (McCarthy *et al*, 1990, McCarthy *et al*, 1991). Indeed it has been suggested that the effects of PGE_2 on bone formation are a secondary consequence of increased production of IGF-I by cells on the osteoblast lineage (Raisz *et al,* 1993, Hakeda *et al,* 1996). The results of recent *in vitro* studies indicate, however, that PGE_2 also has important effects that are independent of IGF-I (Scutt and Bertram, 1995).

The results of studies in rats, and rat bone-derived cells, suggest that PGE_2 acts on a population of primitive inducible osteogenic precursors present at low frequency in the bone marrow (Flanagan and Chambers, 1992, Weinreb *et al,* 1997). Induction of CFU-F colony formation *in vitro* has recently been demonstrated in rat marrow stromal cell cultures (Tang *et al*, 1996), and Scutt *et al* (1995) found that the PGE_2 induced increase in colony formation could be attributed to the recruitment of adherent clonogenic precursors from the non-adherent cell population. These precursors may be distinct from CFU-F, and in a subsequent investigation Scutt *et al* found that the cells which responded to PGE_2 treatment were present in an erythrocyte-enriched subpopulation, rather than from the bone marrow stromal cell subpopulation (personal communication). Recruitment of non-adherent cells was enhanced by the addition of physiological concentrations of dexamethasone, and subsequently it was shown PGE_2-mediated recruitment was mediated via EP_2 receptors (Scutt *et al*, 1995).

The *in vitro* effects of PGE_2 on human bone marrow cells have not been reported in the literature. The influence of PGE_2 on CFE, proliferation and differentiation in cultures of adult human marrow stromal cells has therefore been investigated. Experiments were conducted on whole marrow and three subpopulations of human bone marrow, the wash, the erythrocyte subpopulation (RBC) and the bone marrow stromal cell (BMSC) population. The cell populations were prepared and seeded as in section 2.2.1.1.

The effects of PGE_2 on recruitment of CFU-F from the non-adherent population was also determined. BMSC and RBC subpopulations of bone marrow were explanted into T_{175} flasks at $4x10^5$ cells/cm^2 and incubated overnight at 37°C to allow attachment of the adherent population. Cells in the non-adherent population were recovered after 16 hours by centrifugation and re-cultured at a density of $2x10^4$ cells per cm^2 into petri-dishes (+/- 10^{-8} M Dx) in the absence or presence of 10^{-7} M PGE_2. The non-adherent cells were removed on day 4 and the adherent population cultured for a further 14 days. In some experiments, cultures were treated with PGE_2 in the absence or presence of 10^{-6} M indomethacin from the time of explantation until the removal of non-adherent cells at day 4. As clonogenic cell recruitment was the parameter being investigated it was unnecessary to treat for longer. Thereafter, cells were cultured under standard conditions. The number of clonogenic cells recruited was measured as the number of total and AP positive colonies after 18 days culture.

4.2 Results

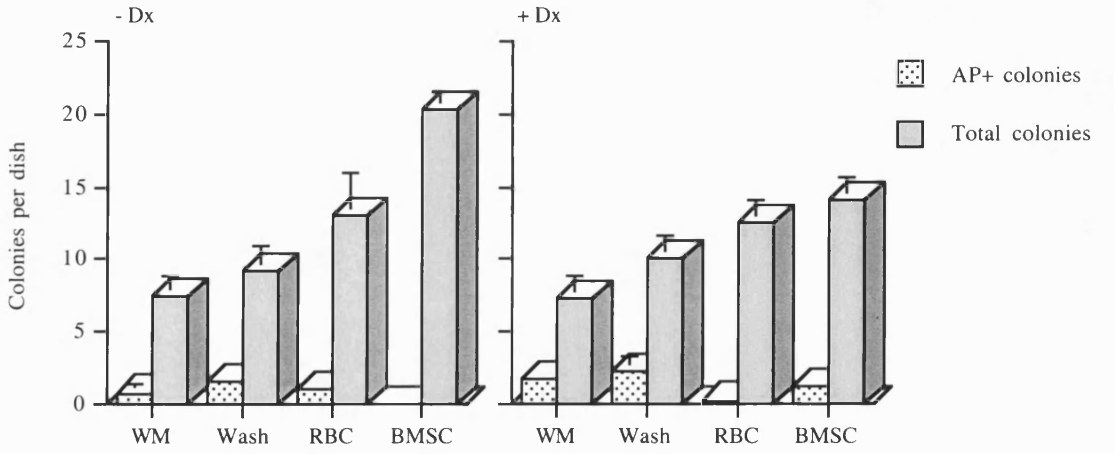
4.2.1 Presence of CFU-F in cell populations of human bone marrow

Figure 4.1 shows colony formation from the different cell populations of human bone marrow, and is representative of 3 experiments using cells from different donors. Colony formation occurred in cultures of each of the marrow subpopulations. In the first series of experiments (figure 4.1i) the greatest number of colonies were derived from the BMSC subpopulation. In this population significantly more colonies were formed than from other marrow cell populations in the absence of Dx, and than from whole marrow in the presence of Dx. Colony formation from the BMSC population was decreased with Dx treatment. Supplementation with Dx induced the formation of colonies expressing AP in all cultures except those of the RBC population. Noticeably, the number of colonies derived from the whole marrow population was not equivalent to the total number of colonies of the subpopulations when combined.

The number of single cells in the cell populations explanted in the experiments shown in figure 4.1i were counted on a haemocytometer. Figure 4.1ii shows an identical series of experiments using cells from different donors in which the number of single cells present in the cell populations were counted electronically with a Coulter Counter (section 2.3.1). This method resulted in an increase in the number of colonies formed from the whole marrow population.

In addition to colonies which formed on the base of the petri-dish, in some cell populations colonies grew on the sides of the dishes, a phenomenon not normally observed in BMSC cultures (figure 4.2). Figure 4.3 shows the number of colonies formed on the sides compared to those on the base of the dishes for the experiment described in figure 4.1i. Colony formation on the sides was particularly abundant in the wash subpopulation, representing ~60% of the total colony formation in this subpopulation. A large proportion of the colonies formed from the whole marrow were also found on the sides of the dish. The majority of these colonies expressed AP. In contrast, fewer than 5% of BMSC colonies formed on the sides.

i)



ii)

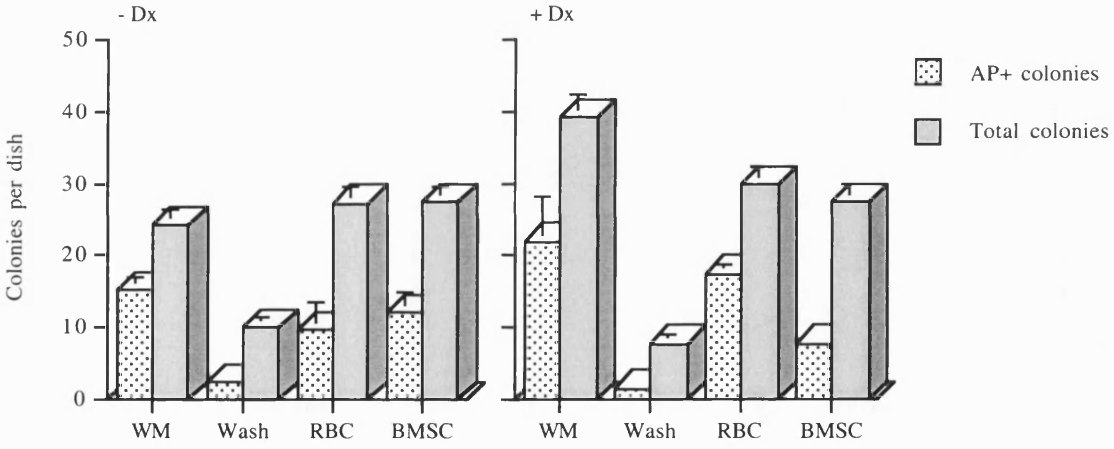


Figure 4.1 Presence of CFU-F in different cell populations of bone marrow

Whole marrow (WM) and the Wash, erythrocyte (RBC), and BMSC populations were cultured under standard conditions +/- 10^{-8} M Dx. Single cells in each cell population were counted prior to explantation (i) using a haemocytometer, and (ii) electronically. After 18 days culture, colonies were fixed in NBF and histochemically stained for AP expression. The results shown are representative of experiments using marrow from (i) n = 3 and (ii) n = 2 donors, and are mean (+/- SE).

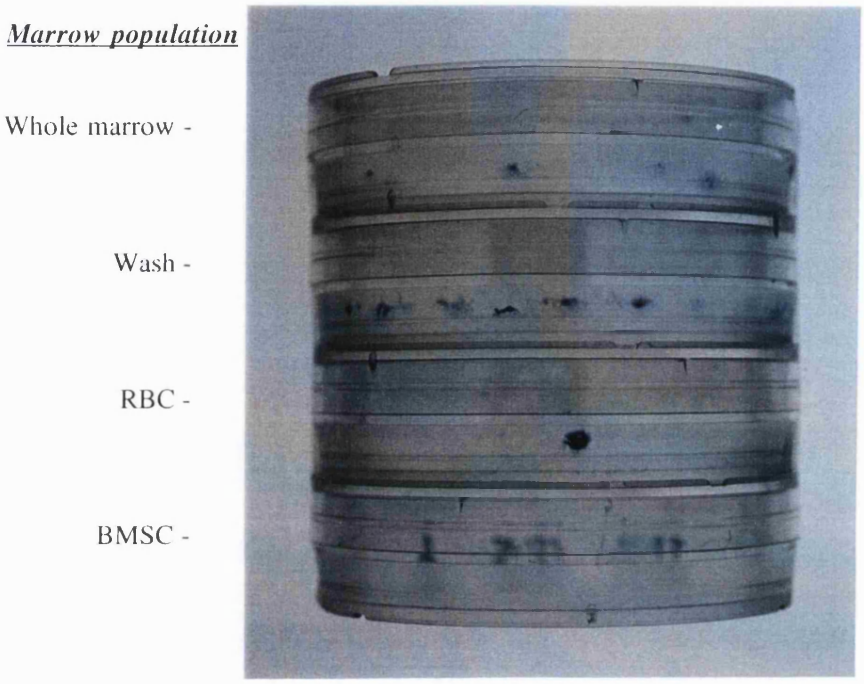
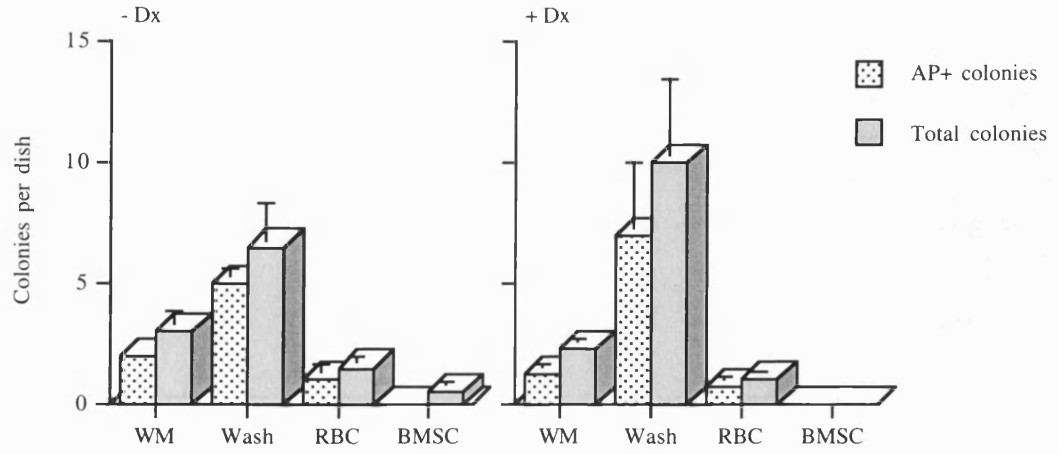


Figure 4.2 Colonies formed on the sides of the dishes in cultures of different cell populations of human bone marrow

Cell populations of bone marrow were cultured for 18 days and colonies were fixed in NBF as described in figure 4.1. Colonies were histochemically stained with Fast-red TR and methylene blue.

i)



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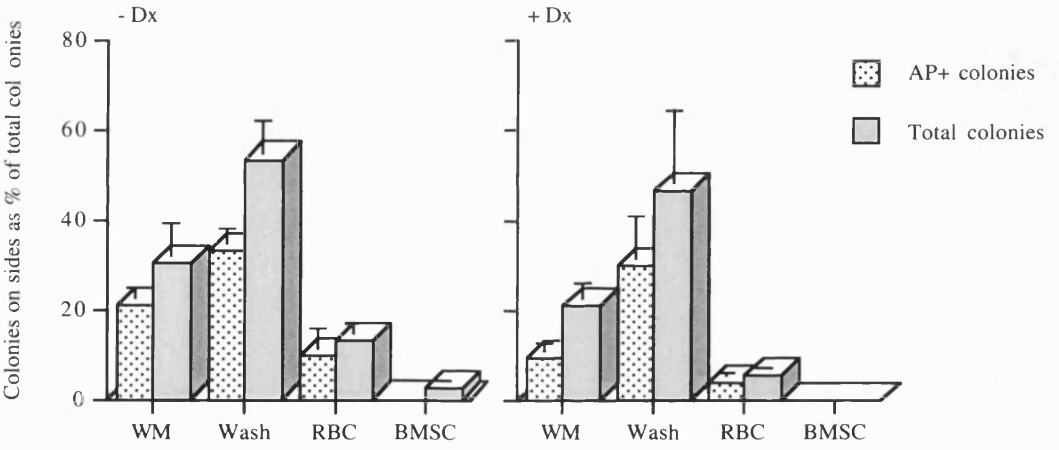


Figure 4.3 The number of colonies formed on the sides of the dishes in cultures of different cell populations of human bone marrow

Cell populations of bone marrow were cultured, and colonies were fixed as described in figure 4.1. Colonies located on the sides of petri-dishes were counted (i), and expressed as a proportion of total colony formation (ii). The results shown are mean (+/- SE) data of one patient sample representative of 3 donors.

4.2.3 Effect of PGE_2 on CFU-F recruitment

The effect of increasing concentrations of PGE_2 on colony formation by bone marrow stromal cells is shown in figure 4.4. The addition of PGE_2 increased total colony formation at $\geq 10^{-7}$ M. At the same doses treatment with PGE_2 also modestly increased the formation of colonies expressing AP, although the proportion of AP positive colonies formed as a % of total remained constant. Cultures were treated with 10^{-7} M PGE_2 in subsequent experiments.

PGE_2 consistently increased colony formation in the presence of Dx in the BMSC population (figure 4.5). However, there was no consistent effect on AP positive colony formation or total colony formation in absence or Dx. When cultures were treated with PGE_2 and indomethacin in combination, total colony formation was reduced in the BMSC population, and unchanged from control in the RBC population (figure 4.6). There was no consistent effect on AP positive colony formation with indomethacin treatment.

In a single experiment the effect of PGE_2 on the production of cyclic adenosine monophosphate (cAMP) from BMSC was investigated. A trend for increased cAMP production was detected at concentrations of $PGE_2 \geq 10^{-8}$ M (figure 4.7).

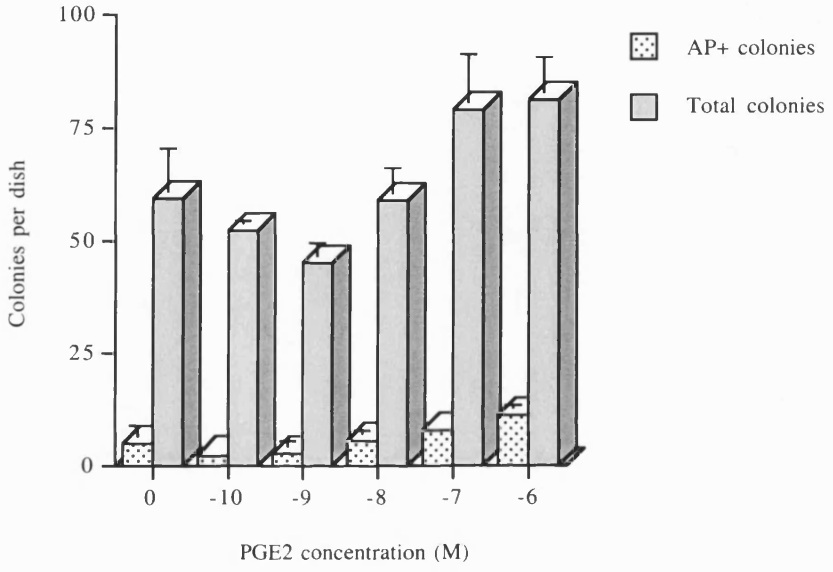
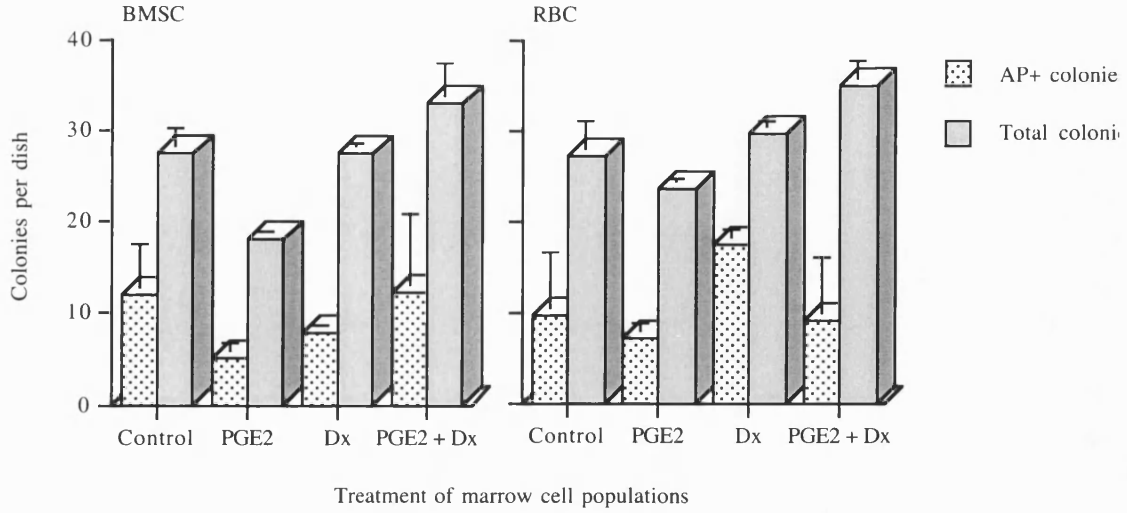


Figure 4.4 The effect of PGE_2 on colony formation in human BMSC

BMSC were cultured with standard medium with 10^{-8} M Dx in the absence or presence of PGE_2. After 18 days, colonies were fixed in NBF and histochemically stained for AP expression. The graph represents 2 experiments using different donor samples and shows mean (n = 4, +/- SE).

i)



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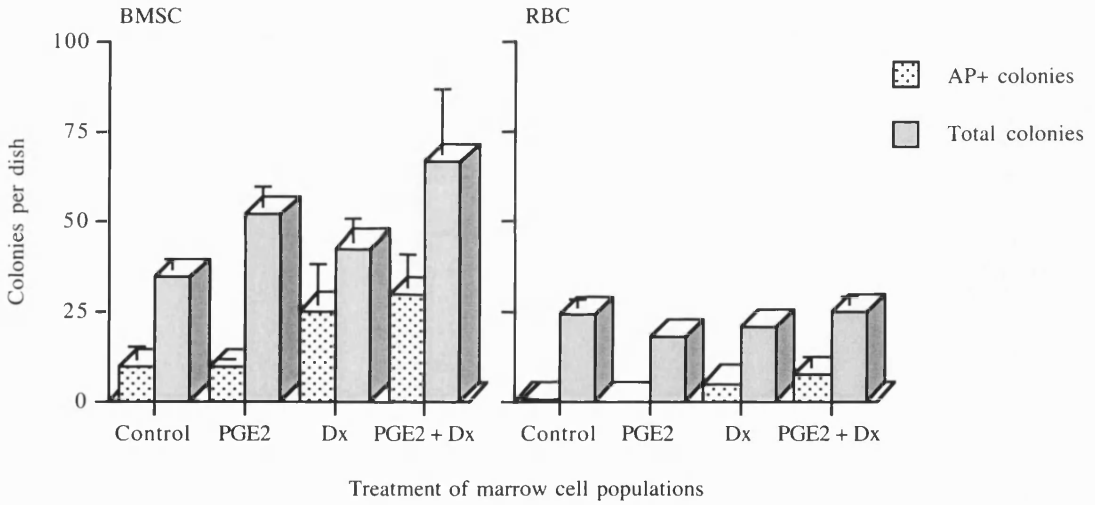

Figure 4.5 Lack of an effect of PGE_2 on colony formation in different cell populations of human bone marrow

Non-adherent cell populations of BMSC and RBC of 2 donor samples (i), and (ii) representative of 4 samples were explanted and cultured in the presence of 10^{-4} M ASP +/- 10^{-8} M Dx, and treated with 10^{-7} M PGE_2. After 18 days the colonies were fixed in NBF and histochemically stained for AP expression. Results show mean (n = 4, +/- SE).

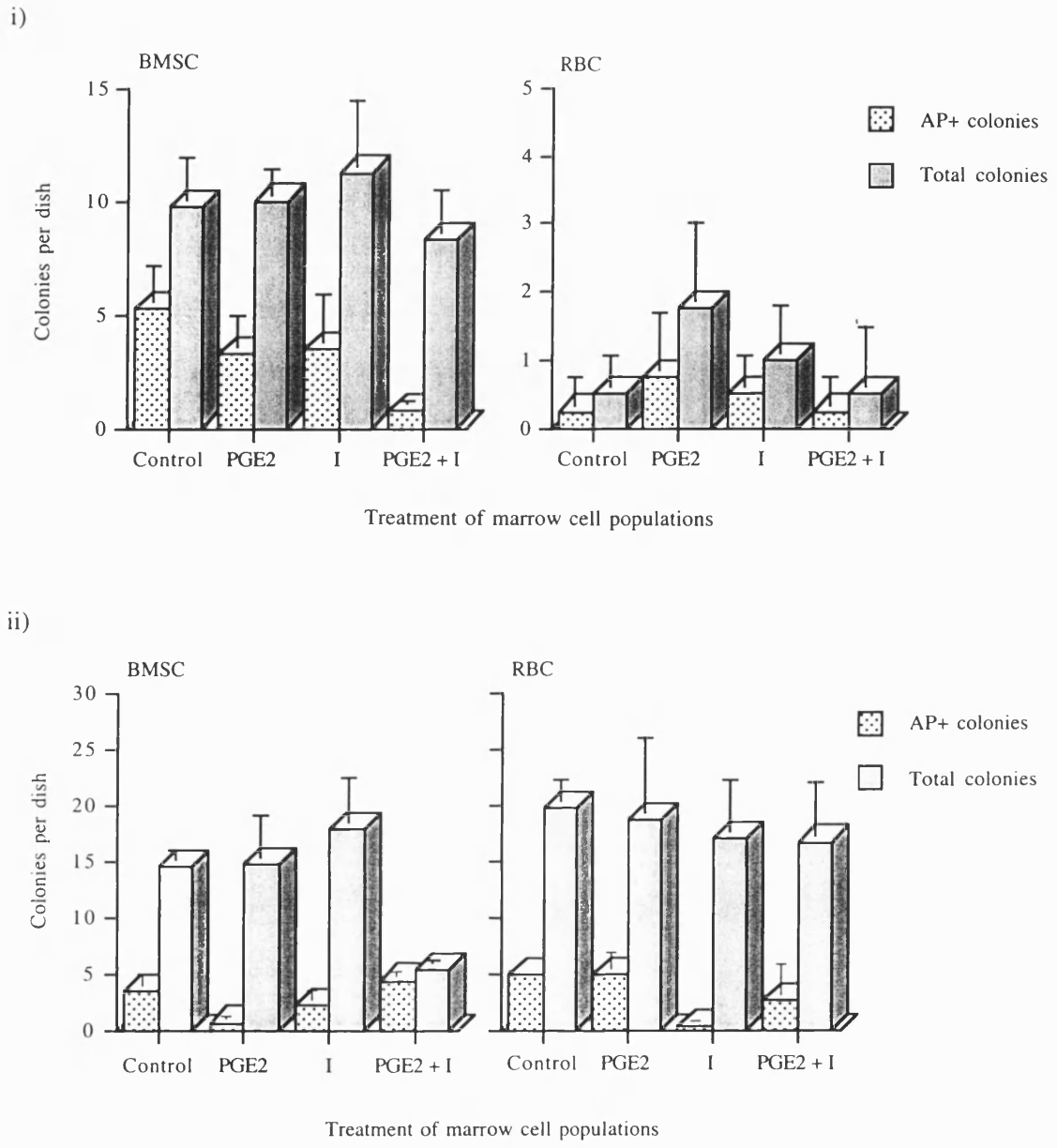

Figure 4.6 The effect of PGE_2 and Indomethacin on CFE in different cell populations of human bone marrow

Non-adherent cell populations of BMSC and RBC of n = 2 donors (i) and (ii) representative of n = 3 donors, were explanted and cultured in the presence 10^{-4} M ASP and 10^{-8} M Dx, and treated with 10^{-7} M PGE_2 and /or 10^{-6} M indomethacin (I). After 18 days the colonies were fixed in NBF and stained for AP. Results show mean (n = 4, +/- SE).

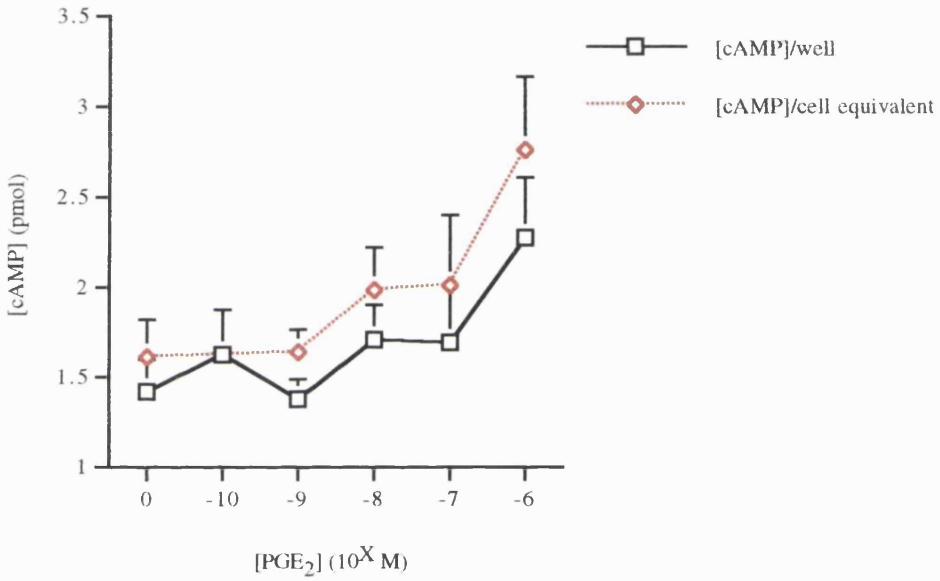

Figure 4.7 Dose response relationship for the stimulation of adenylate cyclase activity by PGE_2 in 2° BMSC

Results of one donor sample showing mean (n = 4, +/- SE). BMSC in 1° culture were treated with various concentrations of PGE_2. Cyclic AMP synthesis resulting from PGE_2 induction was detected as in the methods (section 2.6.6). [cAMP] per cell equivalent indicates the correction of cAMP synthesis for the number of cells in the cultures using a ratio of the optical density of eluted methylene blue stain.

4.3 Discussion

Studies using animal bone marrow have pointed to the existence of an additional class of CFU-F present in the non-adherent population of bone marrow stromal cells and have indicated that their transition from the non-adherent to adherent state is influenced by osteotropic factors including PGE_2 (Flanagan and Chambers, 1992, Scutt *et al*, 1995, Weinreb *et al,* 1997). Results in this study confirm the presence of potential CFU-F in the erythrocyte population of human bone marrow stromal cells, although these were low in abundance. It had been found in the rodent model that this population gave rise to many more colonies than the BMSC population (Scutt *et al*, personal communication). However, this was not the case in these studies. Generally, a greater number of colonies were derived from the BMSC population than from the other cell populations, indicating that the method of density gradient centrifugation enriches CFU-F from the human marrow stromal cell population. However, these results also show that a proportion of clonogenic cells remain present in the wash and RBC populations, and are normally discarded during preparation.

Cell populations of marrow isolated prior to separation of the BMSC population gave rise to colonies on the sides of the dishes. These colonies grew at the level of the meniscus of the medium. It appeared that this population of cells was removed during normal processing, as these colonies were rarely seen in BMSC cultures. It also appeared that the clonogenic cells were of a lower density than the CFU-F which formed colonies on the base of the culture dish. This clonogenic population gave rise to smaller, less proliferative colonies than those on the base, which were largely AP positive. This may demonstrate further, that the CFU-F population are not only multipotential, but are also heterogeneous in phenotype (Rickard *et al*, 1994, Long *et al*, 1995). In effect, buoyant cells with clonogenic capacity may become trapped in the surface tension of the meniscus of the medium and adhere to the sides of the dish. In this case, cellular differentiation might be enhanced as a result of increased cell density, due to restricted cellular migration and the limited expansion area. Although AP positive, further studies would be necessary to confirm that these colonies contain cells of the osteoblast lineage. Also of interest is the specific density of these clonogenic cells which could be determined by isopycnic density gradient centrifugation.

Interestingly, the number of colonies formed from whole marrow did not equal the number derived from the BMSC population, or even the total number of colonies from the wash, RBC and BMSC populations, and represented 17% (19% with Dx) of the colony formation of the combined subpopulations. This may be due to the influence of other stromal and haematopoietic cell types present in the cultures as described in section 3.3. This discrepancy in colony formation may have resulted also from the difficulty to accurately determine the absolute number of mononuclear cells in whole marrow.

Results in this study also confirm the presence of potential CFU-F in the non-adherent population of human bone marrow stromal cells. The presence of CFU-F in the non-adherent population has previously been shown in rat (Scutt and Bertram, 1995) and human (section 3.3, Oreffo *et al*, 1995) marrow stromal cultures. No evidence was found to confirm that exogenous PGE_2 can influence transition of cells from the non-adherent population to become adherent human BMSC, as had been suggested by Scutt *et al* (1995) in the RBC population. PGE_2 induced a moderate increase in colony formation in the BMSC population.

The importance of endogenous PGE_2 on colony formation was assessed by the addition of dexamethasone or indomethacin to cultures. Exogenous glucocorticoids inhibit PGE_2 synthesis through down-regulation of PLA_2 transcription and induction of lipocortin-1 synthesis, an anti-inflammatory peptide mediator which inhibits the activity of PLA_2 (Rang *et al*, 1995). Osteoblasts grown with dexamethasone *in vitro* have been shown to synthesise significantly lower endogenous levels of PGE_2, resulting in interference of osteoblast proliferation (Hughes-Fulford *et al*, 1992). Inducible prostaglandin G/H synthase is inhibited by NSAIDS such as indomethacin (Pilbeam *et al*, 1995).

Cultures treated with Dx provided no evidence of an effect of endogenously produced prostaglandins on colony formation, although, inhibition of PGE_2 synthesis with indomethacin tended to increase colony formation in the BMSC population, and addition of exogenous prostaglandin to indomethacin-treated cultures reduced the number of colonies formed. A larger experimental sample number is necessary to confirm this observation. In addition, incubation of the cells in serum-free medium may have elicited a greater response, and pre-treatment of the cultures with the anti-inflammatory compounds would be worthwhile to inhibit endogenous prostaglandin synthesis prior to the addition of exogenous prostaglandin.

The inability to detect an effect of PGE_2 on recruitment of CFU-F from the non-adherent population may be the result of a species difference. Differences between colony formation of rat and human osteoprogenitors have previously been described (Beresford *et al,* 1993, Lian *et al,* 1997, Kuznetsov and Robey, 1997), and variations in these experiments may result from differences between the ability of clonogenic cells from either species to respond to PGE_2 treatment. However, differences may also occur due to the differences in skeletal site from which the sample was taken. For instance, marrow from human donors was taken from rib, a skeletal site which is placed under relatively little strain and therefore may not be as responsive to stimulation by factors such as prostaglandins. Marrow samples from rodents however, were taken from the femur, a site which is constantly being placed under tension and compression.

This may also reflect discrepancies in the experimental design. For example the dose response curve indicates that the concentration of 10^{-7} M PGE_2 used was possibly too low and 10^{-6} M would have been more appropriate. An additional factor is that most of the donors used in this study were diagnosed as suffering from lung cancer (appendix III). Chasseing *et al* (1997 and 1997a) have shown recently that endogenous PGE_2 is overproduced by stromal colonies derived from the marrow of patients suffering with lung and advanced breast cancers, and suggest that this may account for the low CFE of marrow from this group of patients.

Chapter 5

The effect of PDGF on the growth of human CFU-F

5.1 Introduction

The aim of the initial phase of this investigation was to define the basic conditions for the isolation of BMSC and their establishment in culture. This having been achieved, the primary objective became the identification of one or more growth factors that would support *ex-vivo* expansion of the stromal cells without influencing their potential for osteogenic differentiation.

Platelet-derived growth factor (PDGF) is a potent, naturally occurring growth factor known to be mitogenic for a variety of mesenchymal cell types (Ross *et al*, 1974, Heldin *et al*, 1992) including cells of the osteoblast lineage. When compared directly, PDGF was suggested to be more mitogenic for human cells of the osteoblast lineage than TGFβ, IGF-I, or EGF (Piche and Graves, 1989), and so was selected for further investigation in our culture system.

PDGF was originally identified as a factor released from the α-granules of platelets during aggregation and clot formation (Ross *et al*, 1974, Kohler and Lipton, 1974). However, PDGF is also produced by the more mature cells of the osteoblast lineage (Graves *et al*, 1989, Zhang *et al,* 1991, Rydziel *et al,* 1992, Andrew *et al*, 1995, Horner *et al*, 1996) and is sequestered in bone matrix (Hauschka *et al*, 1986). The matrix bound PDGF fraction is thought to be released during bone resorption and then act as a paracrine regulator of the chemotaxis, proliferation and biosynthetic activity of osteoblastic cells (Centrella *et al*, 1989, Pfeilschifter *et al,* 1990, Gilardetti *et al*, 1991). PDGF is secreted as disulphide bonded homo or heterodimer of closely related A and B polypeptide chains, forming PDGF AA, BB and AB (Heldin *et al*, 1992). The actions of PDGF are mediated via interaction with two different receptor types, termed PDGF receptor α subunit (PDGFRα) and PDGF receptor β subunit (PDGFRβ). Binding of divalent PDGF induces dimerisation of the single transmembrane glycoproteins with three possible configurations αα, ββ and αβ, which then transduce a cell response through intracellular tyrosine kinase activity. Both A and B peptides are high affinity ligands for PDGFRα, whereas the B chain only interacts with PDGFRβ. Ligand interaction with both receptor types induces a mitogenic response, but only occupancy of the β-receptor can induce actin reorganisation and chemotaxis (Heldin *et al*, 1992). Receptors are widely distributed on cells of mesenchymal origin including fibroblasts and cells of the osteoblastic lineage (Bryckaert *et al*, 1988, Gilardetti *et al*, 1991).

Administration of PDGF *in vivo* increases bone mineral density and strength in rats (Mitlak *et al*, 1996), and stimulates endosteal and periosteal bone formation in rabbits (Nash *et al*, 1994). It is known however, that the PDGF isoforms differ in their functional properties and studies using the homo and heterodimers have shown that those containing B chain

subunits have the greatest mitogenic potential for cells of mesenchymal origin (Kelly *et al*, 1985, Centrella *et al*, 1991).

In this chapter the influence of continuous PDGF treatment on the proliferation and differentiation of CFU-F derived from adult human BMSC has been investigated. Experiments were performed to determine the effects of PDGF AA and BB homodimers on colony forming efficiency, and on the subsequent proliferation and osteogenic differentiation of the adherent cell population.

5.1.1 Interaction of PDGF with IGF-I responsive pathways

IGF-I is also an important anabolic growth factor in bone (Delany *et al*, 1994). It is expressed by mature osteoblasts *in vivo*, and stimulates osteoblast cell proliferation and protein synthesis *in vitro* (Middleton *et al*, 1995, Shinar *et al*, 1993, Wang *et al*, 1995). Although IGF-I and PDGF are able to induce mitogenic effects on osteoblasts independently they have been found to produce a synergistic effect when added in combination (Canalis *et al*, 1989, McCarthy *et al*, 1989, Delany *et al*, 1994, Tanaka and Liang, 1995). The PDGF and IGF-I receptors (IGF-IR) are both tyrosine kinase regulators and share a common transduction pathway. In addition, it has been suggested that the growth factor response is not independent of IGF-IR transduction (DeAngelis *et al,* 1995). In transfectants which over-express the PDGFRβ but lack the IGF-IR, it has been shown that PDGF BB cannot induce mitogenesis or transformation. Thus, it appears that the ability of the activated β receptor to stimulate cell proliferation and transformation requires a functional IGF-IR (DeAngelis *et al,* 1995).

It was of interest, therefore, to investigate whether the effects of PDGF in this system were mediated in whole or in part by IGF-I. The effects of low and high concentrations of IGF-I on BMSC proliferation were studied alone, and in combination with PDGF BB.

5.2 Results

5.2.1 Synthesis of platelet-derived growth factor by bone-derived cells

The ability of BMSC to synthesise PDGF endogenously was investigated by RT-PCR (figure 5.1). The human osteosarcoma cell line MG-63, was included as a negative control as it has been reported to not produce PDGF (Hauschka *et al,* 1988). A and B chain transcripts were detected in cultures derived from both donors and, albeit at much lower levels in the MG-63 cells. Treatment with Dx increased PDGF A chain transcript expression in MG-63 cells, but had no consistent effect on its expression in the normal cells. In contrast, treatment with Dx, decreased B chain transcript expression in all normal cell cultures studied. In MG-63 cells, however, its expression was increased.

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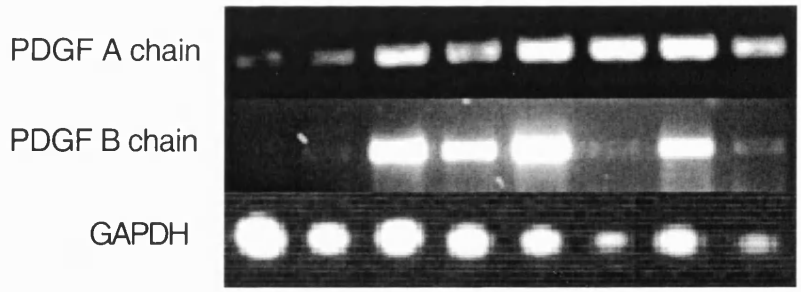

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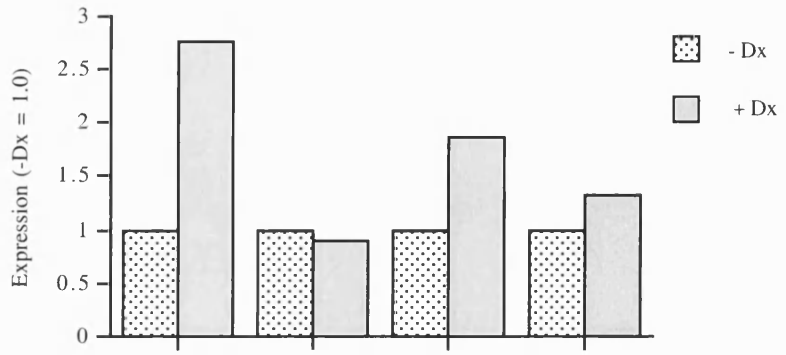


iii)

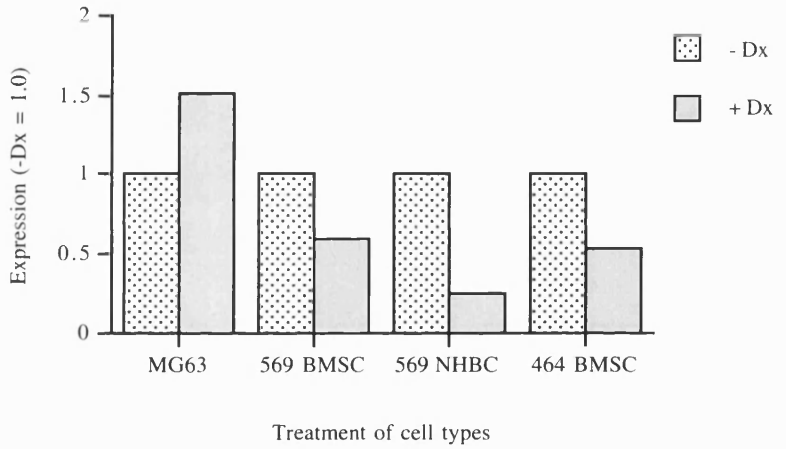


Figure 5.1 Expression of PDGF A and B mRNA in cultures of human bone-derived cells

cDNA products in a 1.8% ethidium bromide stained agarose gel (i), after RT-PCR amplification of mRNA using primers specific for PDGF A and B chains, and GAPDH. mRNA was extracted from whole lysates of MG-63 cells, BMSC and HBDC cells of donor #569, and BMSC of donor #464. Note that this method does not allow a direct comparison to be made between the levels of PDGF A and B transcripts. Relative expression of PDGF A (ii) and PDGF B (iii) when cultured in the absence or presence of Dx in the different cell types. The data have been normalised for the amount of GAPDH to correct for variation in the amount of input mRNA. Cycle number was 35 for all reactions.

5.2.2 Expression of PDGF α and β receptor chains

Greater than 95% of the MG-63 cells expressed receptors for PDGF isoforms (figures 5.2i - iv). α and β receptors were expressed by fewer cells obtained from marrow or trabecular explants, although the surface density of receptors on normal bone cells was greater than on MG-63 cells. When medium was supplemented with Dx the number of normal bone-derived cells expressing α receptors was reduced, whereas the number of receptor sites per cell was not altered. The number of cells expressing the PDGF β receptor was reduced in BMSC with Dx treatment, and increased in HBDC. The surface density of the receptors was also reduced on BMSC, but on HBDC was unaffected. In the donor samples investigated, surface expression of PDGFRα was similar on BMSC and HBDC, whereas BMSC expressed greater numbers of PDGFRβ at the cell surface.

		BMSC	HBDC
% total	PDGFRα	38.3% (+/- 5.8) - 69.9% (+/- 9.6)	47.7% (+/- 5.4)
	PDGFRβ	48.8% (+/- 11.3)	36.5% (+/- 5.4)
MFI	PDGFRα	44.7 (+/- 0.4) - 31.6 (+/- 2.4)	36.6 (+/- 1.1)
	PDGFRβ	52.8 (+/-15.7)	47.8 (+/- 3.9)

Table 5.2iv PDGF receptor chain expression on cultured human bone-derived cells: Ranges for duplicate donors and mean values for single or multiple donors

Analysis by western blotting confirmed that the antibodies used in FACS analyses recognised the PDGF receptor chain isoforms (figure 5.3). These were detected as 180 kDa bands after electrophoretic separation. No change in receptor peptide expression was detectable by western blotting with Dx treatment. The presence of other bands visible on the blot after immunolabelling was due to non-specific antibody binding and the presence of products of receptor degradation.

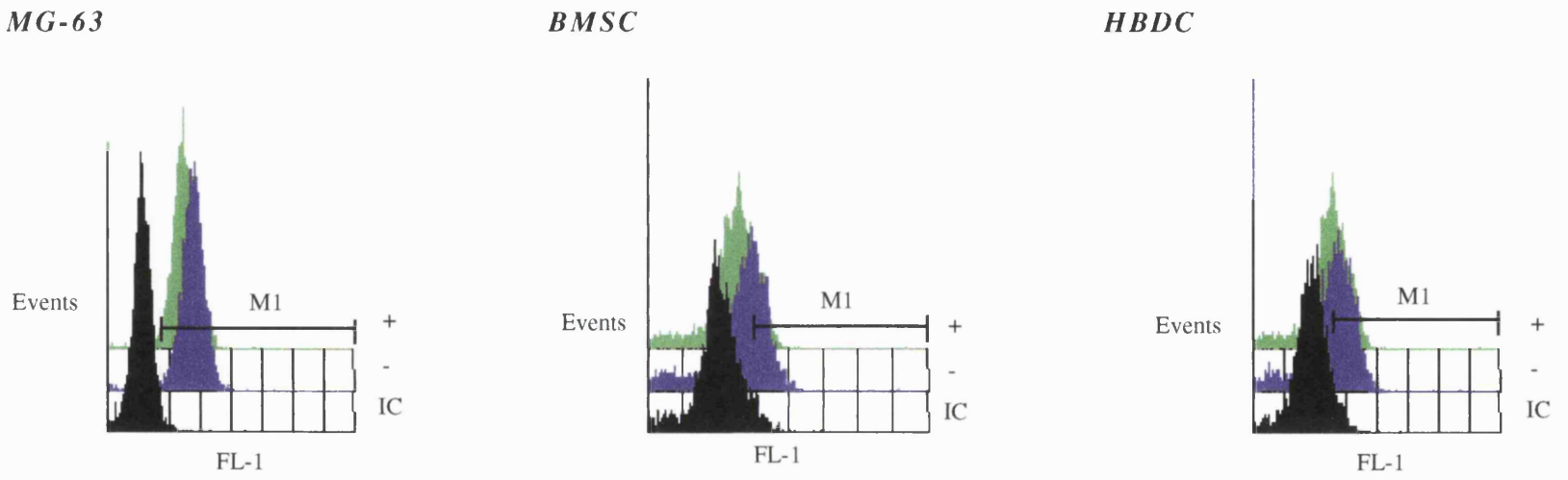


Figure 5.2i The expression of PDGF α receptor chain by human bone-derived cells

Cells at first passage were cultured in standard medium in the absence or presence of 10^{-8} M Dx (-/+) for 5 days. At the end of this period the cells were harvested by sequential collagenase-trypsin treatment and then immunolabelled using a purified rabbit anti-PDGF α receptor chain, followed by a FITC-conjugated 2° antibody. Results of peak shift in fluorescent intensity (FL-1, log scale) generated from a 5% marker (M1) on the negative control (cell labelled with isotype matched control antiserum, IC) histogram are shown in figure 5.2iii. The data shown are representative of 1, 2 and 1 experiments for MG-63, BMSC and HBDC respectively.

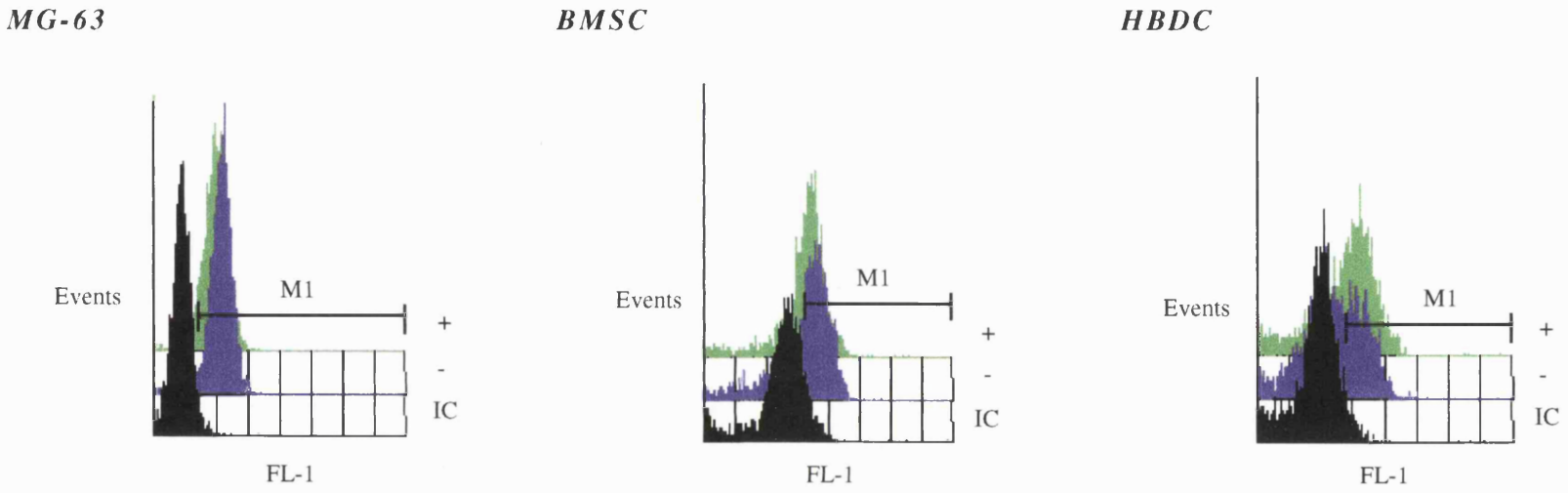


Figure 5.2ii The expression of PDGF β receptor chain by human bone-derived cells

Cells at first passage were cultured in standard medium in the absence or presence of 10^{-8} M Dx (-/+) for 5 days. At the end of this period the cells were harvested by sequential collagenase-trypsin treatment and then immunolabelled using a purified rabbit anti-PDGF β receptor chain, followed by a FITC-conjugated 2° antibody. Results of peak shift in fluorescent intensity (FL-1, log scale) generated from a 5% marker (M1) on the negative control (cell labelled with isotype matched control antiserum, IC) histogram are shown in figure 5.2iii. The data shown are representative of 2, 6 and 1 experiments for MG-63, BMSC and HBDC respectively.

PDGF α

Cell type	Treatment	Marker	Events	% Total	MFI
MG63	-	All	5000	100.00	22.94
		M1	4868	97.36	24.49
MG63	+	All	5000	100.00	16.61
		M1	4813	96.26	18.02
BMSC	-	All	5000	100.00	21.94
		M1	2049	40.98	45.08
BMSC	+	All	5000	100.00	16.90
		M1	1022	20.44	41.84
NHBC	-	All	5000	100.00	18.73
		M1	2384	47.68	35.73
NHBC	+	All	5000	100.00	16.90
		M1	1874	37.48	34.01

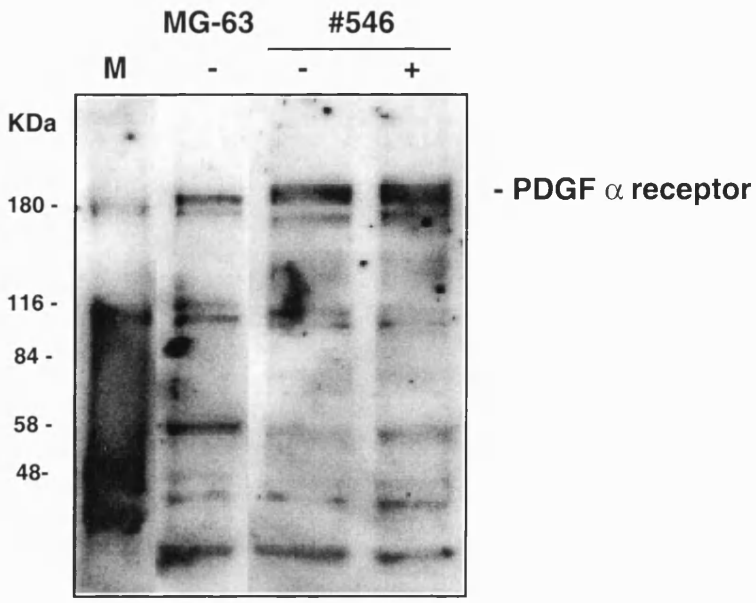
PDGF β

Cell type	Treatment	Marker	Events	% Total	MFI
MG63	-	All	5000	100.00	11.58
		M1	4833	96.66	12.34
MG63	+	All	5000	100.00	8.85
		M1	4563	91.26	9.90
BMSC	-	All	5000	100.00	42.95
		M1	2660	53.20	84.24
BMSC	+	All	5000	100.00	38.20
		M1	2070	41.40	74.80
NHBC	-	All	5000	100.00	15.77
		M1	1826	36.52	47.80
NHBC	+	All	5000	100.00	22.78
		M1	2776	55.52	49.01

Table 5.2iii PDGF receptor chain expression on cultured human bone-derived cells

Table showing the results of flow cytometric analyses of cells immunolabelled with antibodies against PDGFRα and PDGFRβ chains. Experimental details were as described in the legend to figures 5.2i and 5.2ii, where cells were cultured +/- Dx (+,-). M1 shows the results of a 5% margin marker on the negative control histograms in figures 5.2i and 5.2ii. The MFI (mean fluorescent index) is an arbitrary indication of the fluorescent intensity per cell.

i)



ii)

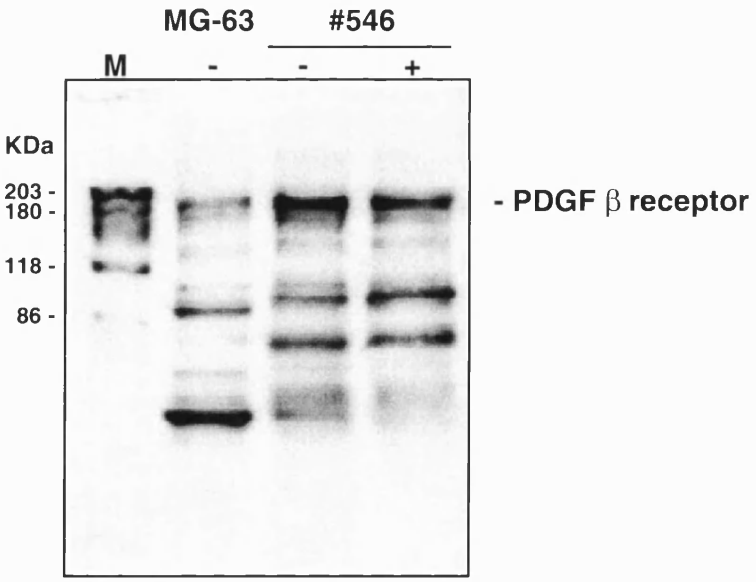


Figure 5.3 Identification of PDGF receptor protein chains

Whole cell lysates from BMSC of donor sample #546, cultured in the absence or presence of Dx (-/+), and MG-63 cells, were electrophoresed on 7.5% polyacrylamide gels and western blotted. Following incubation with primary antibody and peroxidase-conjugated secondary antibody, the receptor chains were visualised using enhanced chemiluminesence. M, molecular weight markers.

5.2.3 The influence of PDGF on BMSC proliferation: dose response

In the absence of Dx, treatment with PDGF BB (0.1 - 2.5 ng/ml) resulted in an increase in cell proliferation (figure 5.4). In the presence of Dx, the response was more clearly dose dependent although the maximal effect was observed over the same dose range (1-5 ng/ml). Treatment with 2.5 ng/ml PDGF BB induced a significant response in the absence or presence of Dx ($p < 0.05$). In other experiments using cells from different donors, the maximal increase was found to occur reproducibly at 2.5 ng/ml (4.0 +/- 2.0 fold for $n = 3$ donors). In contrast to the PDGF BB isoform, treatment with PDGF AA over the same dose range was found not to affect BMSC proliferation in the absence of Dx. In the presence of Dx, however, a significant increase in proliferation was observed at a dose of 2.5 ng/ml (figure 5.5). On the basis of these observations, PDGF BB and AA were used at 2.5 ng/ml in all subsequent experiments.

5.3 The effect of PDGF on CFU-F growth

5.3.1 CFE

Colonies formed in primary cultures of human bone marrow-derived stromal cells, cultivated in the absence or presence of 10^{-4} M ASP, 10^{-8} M ASP, DX or/and 2.5 ng/ml PDGF BB are shown in figure 5.6. Neither the PDGF BB or AA isoforms affected total CFE at the dose tested (2.5 ng/ml, figures 5.7 and 5.8). In contrast, with a single exception (PDGF BB + ASP + Dx), the number of AP positive colonies was reduced, though not significantly, with AA or BB treatments under all conditions. This reduction resulted from a decrease in the proportion of AP positive colonies formed.

5.3.2 Proliferation

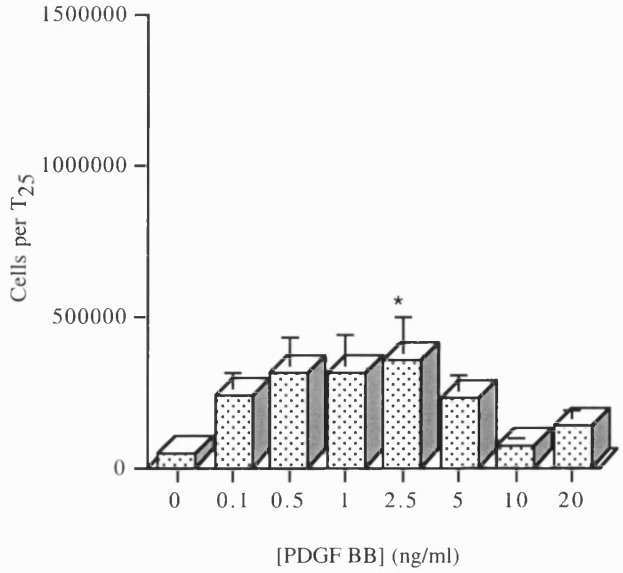
5.3.2.1 Colony size

Treatment with both PDGF homodimers increased the mean area per colony (figure 5.9). The large error bars on these histograms demonstrate the large deviation in colony size between patient samples. The greatest increase in colony size in response to PDGF treatment occurred in the presence of Dx.

5.3.2.2 Cell number

Treatment of BMSC cultures with PDGF BB and AA induced cellular proliferation, whether added alone or combined with ASP or/and Dx (figure 5.10). The largest effect on proliferation was observed when the cells were treated with PDGF BB or AA in the presence of ASP and Dx. PDGF BB treatment had a slightly greater effect on proliferation than PDGF AA. Whilst a significant increase in proliferation was shown in figure 5.4, no significant effect was established when results from a number of donors were pooled.

i)



ii)

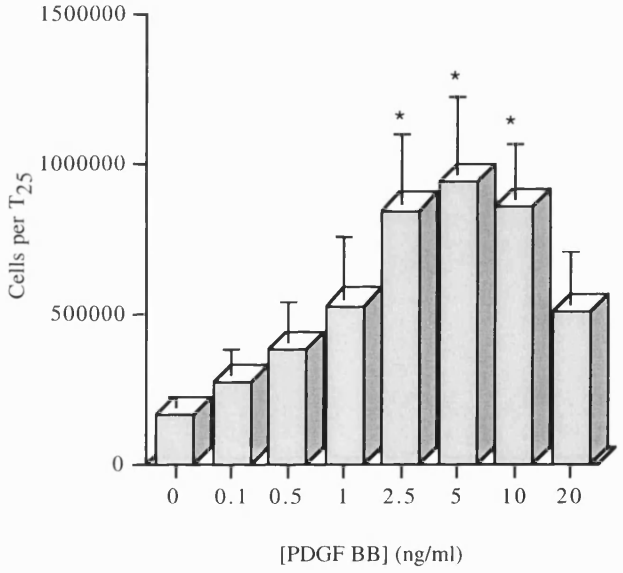
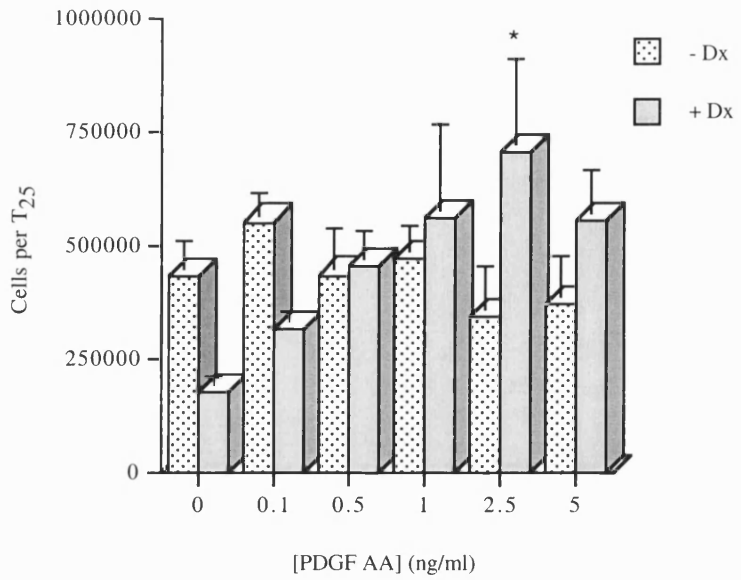


Figure 5.4 Dose response for the effect of PDGF BB on BMSC proliferation

Results of one donor sample representative of three, showing mean (n = 4) +/- SE. BMSC in 1° culture were treated with various concentrations of PDGF BB, in the absence (i) and presence (ii) of 10^{-8} M Dx for 28 days. Cells were harvested by treatment with sequential collagenase-trypsin and counted electronically. * Indicates a significant increase in cell number over control cultures (0 ng/ml PDGF, $p < 0.05$).

i)



ii)

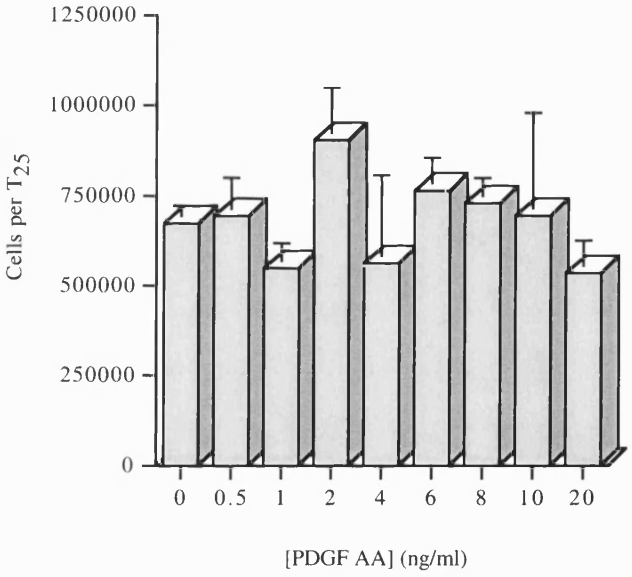


Figure 5.5 Dose response for the effect of PDGF AA on BMSC proliferation

Cultures of BMSC from two donor samples (i and ii) were treated with various concentrations of PDGF AA for 28 days in the absence or presence of 10^{-8} M Dx (ii = + ASP and Dx). Cells were harvested by treatment with sequential collagenase-trypsin and counted electronically. Results show mean (n = 4) +/- SE, where * indicates a significant increase in cell number over ASP + Dx control culture ($p < 0.05$).

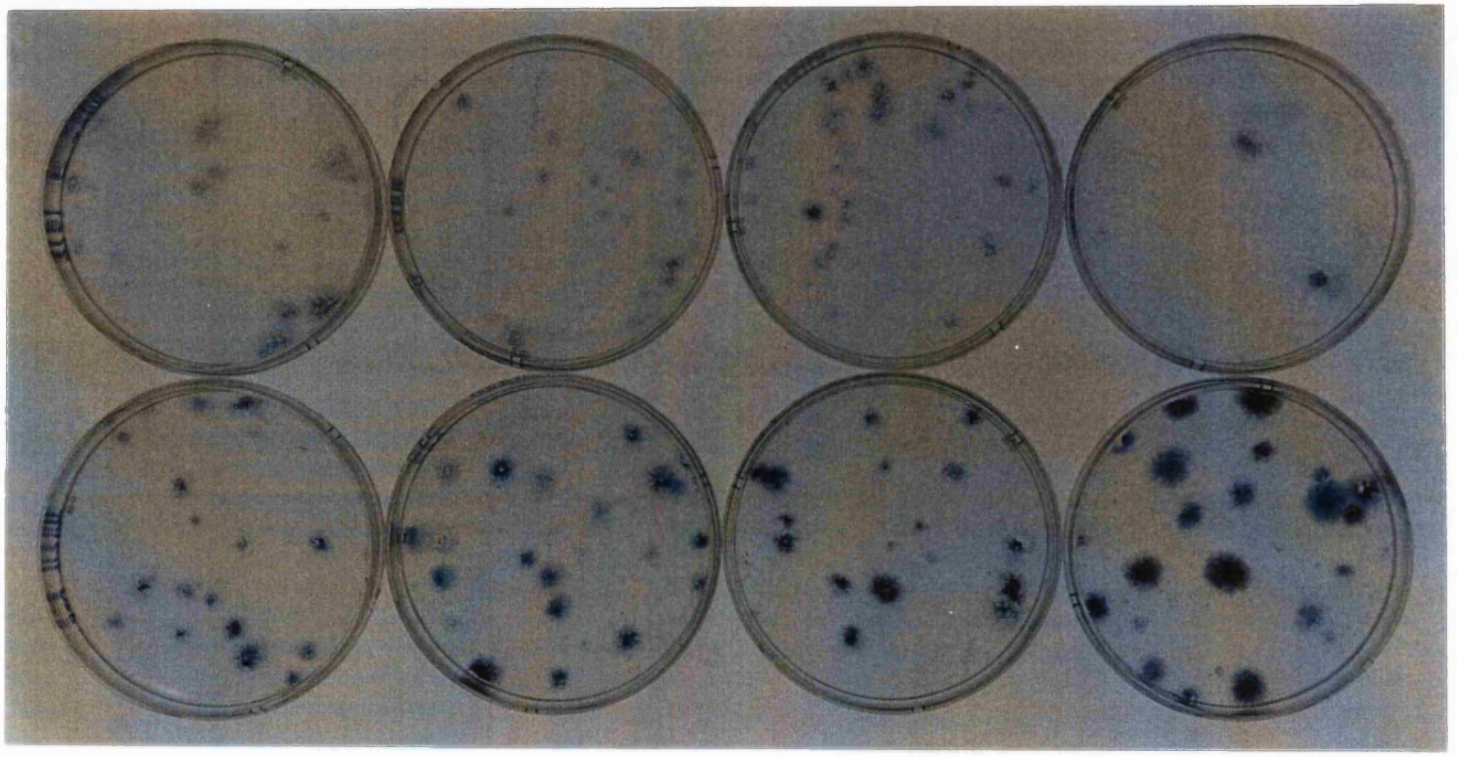
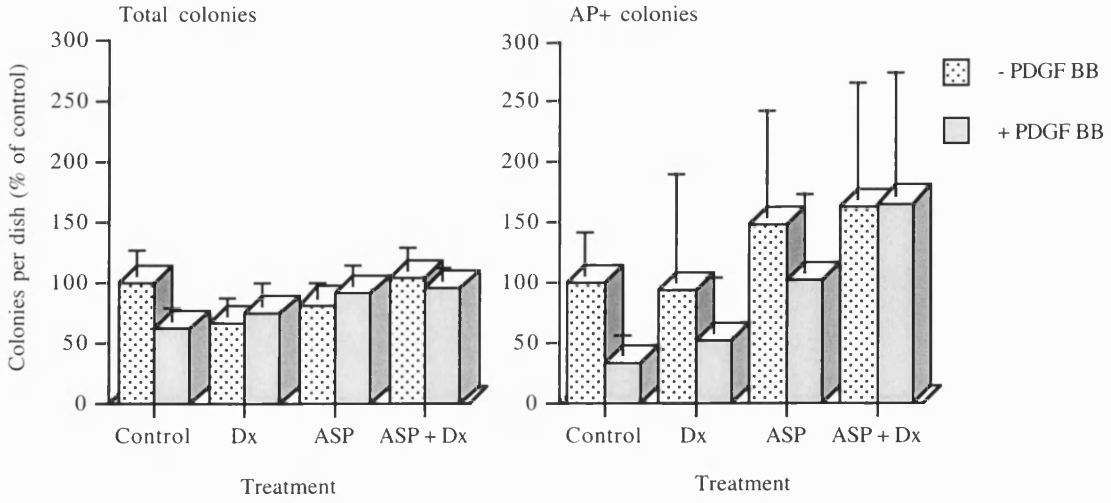


Figure 5.6 The effect of PDGF BB on colony formation by human marrow-derived bone cells

BMSC were cultured in the absence or presence of ASP, Dx and/or PDGF BB under standard 1° culture conditions for 18 days. Cultures were fixed with NBF, then stained sequentially with Fast-red TR and methylene blue stains.

i)



ii)

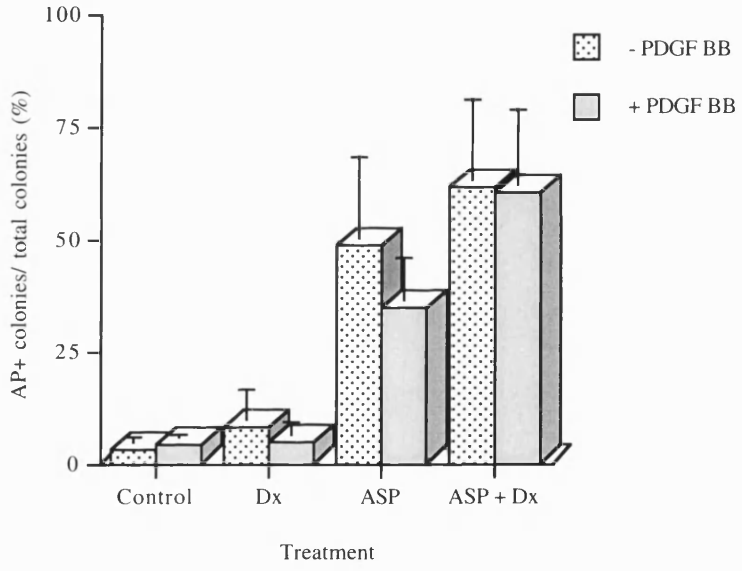
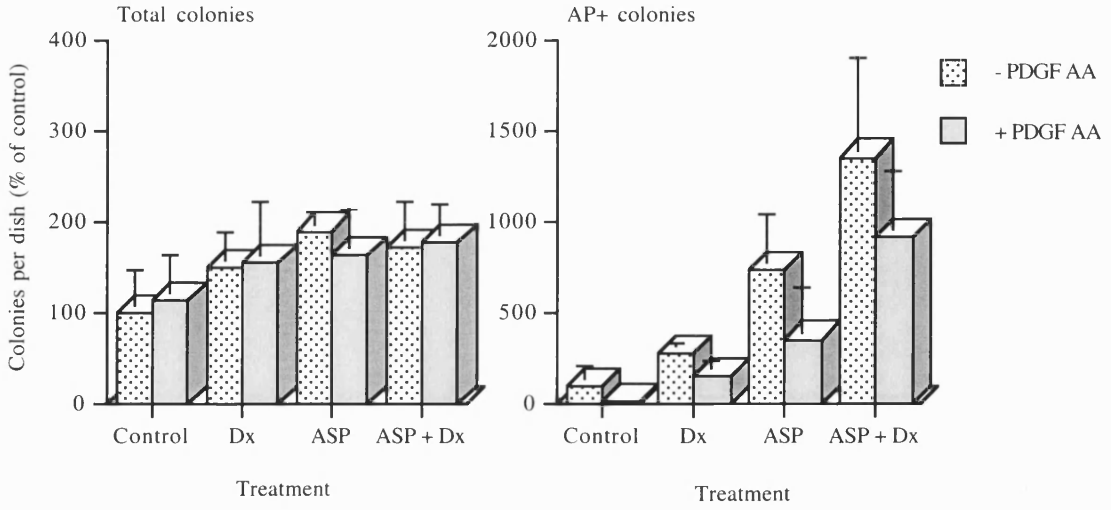


Figure 5.7 The effect of PDGF BB on CFE in cultures of human BMSC

BMSC were treated with 2.5 ng/ml PDGF BB, alone or in combination with 10^{-4} M ASP and 10^{-8} Dx. Colonies were fixed with NBF on day 18 and histochemically stained for AP and counter stained with methylene blue. Data have been expressed as percentage of the total number and number of AP+ colonies formed in the control (-Dx, -ASP) cultures (i), and AP+ colonies as a percentage of the total colonies formed (ii). Results shown are the mean +/- SE for n = 6 donors. Control cultures contained 15.8 (+/- 4.4) total, and 11.12 (+/- 4.7) AP+ colonies respectively (+/- SE).

i)



ii)

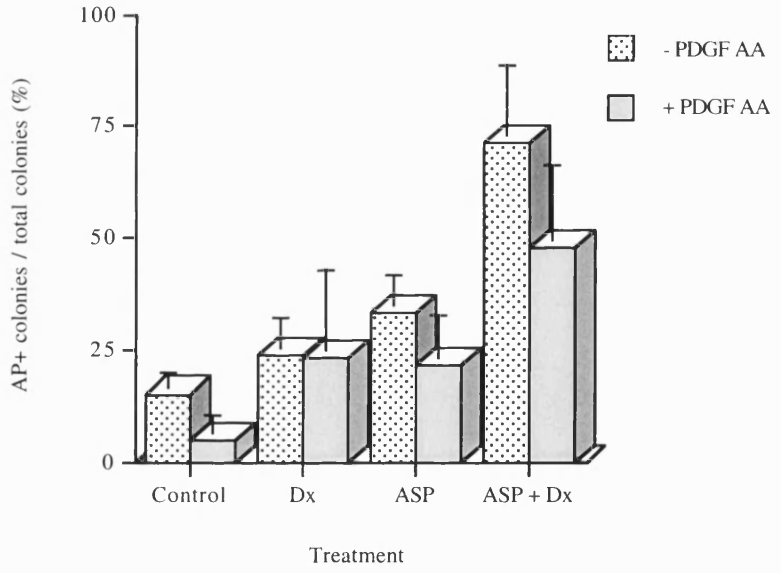
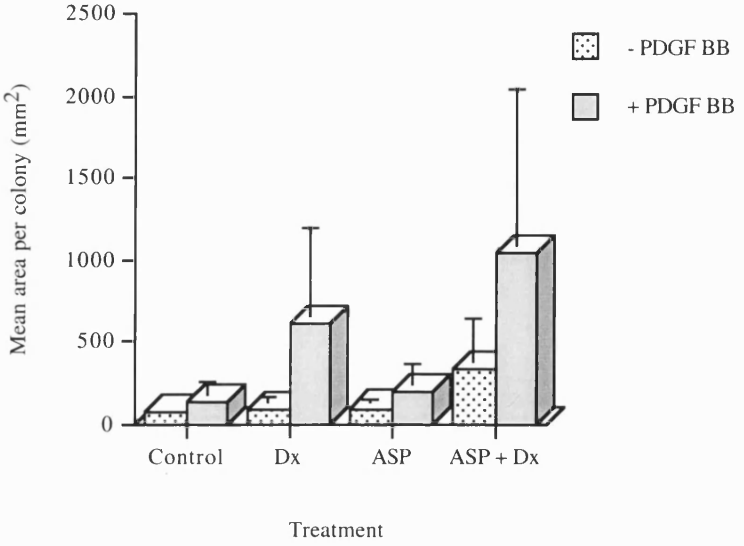


Figure 5.8 The effect of PDGF AA on CFE in cultures of human BMSC

BMSC were cultured with 2.5 ng/ml PDGF AA and stained as described in the legend to figure 5.7. Data have been expressed as percentage of the total number and number of AP+ colonies formed in the control (-Dx, -ASP) cultures (i), and AP+ colonies as a percentage of the total colonies formed (ii). Results shown are the mean +/- SE for n = 4 donors. Control cultures contained 15.6 (+/- 7.5) total, and 1.1 (+/- 1.3) AP+ colonies respectively (+/- SE).

i)



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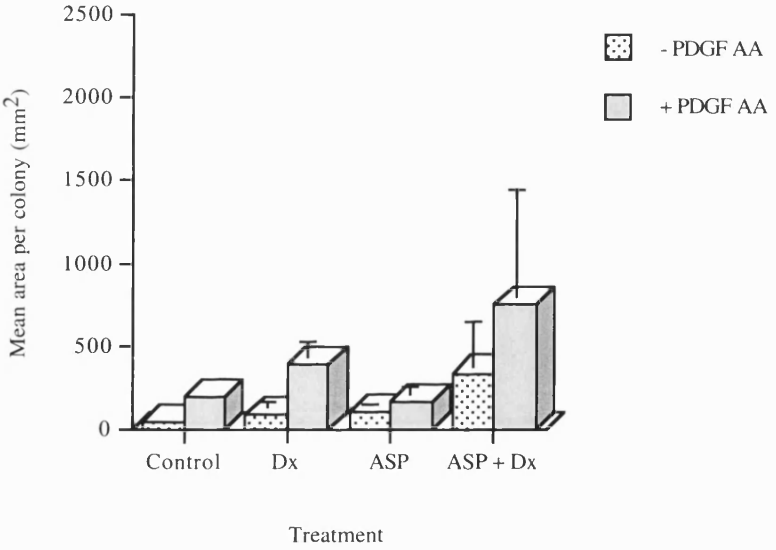
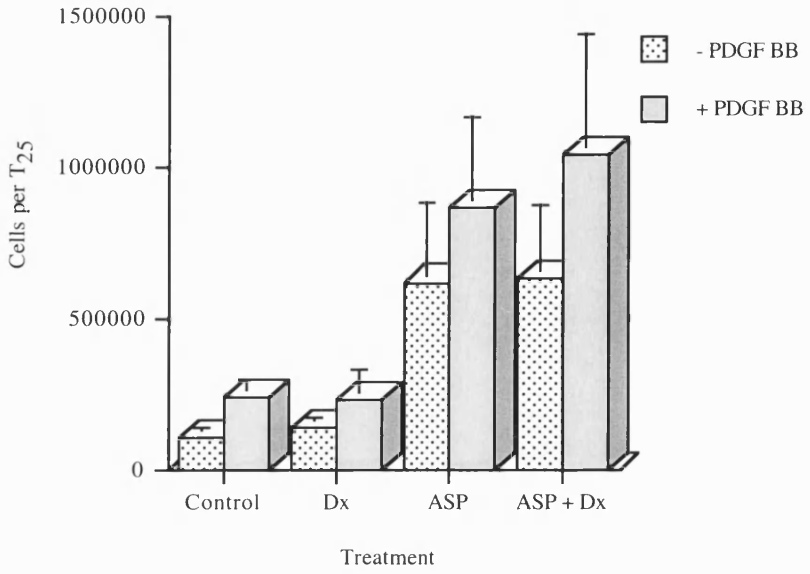


Figure 5.9 The effect of PDGF on colony area in cultures of human BMSC

BMSC were cultured and treated with PDGF BB (i) or AA (ii) as described in figure 5.7. The colony diameter was measured (mm) and the mean area of the colonies under each treatment calculated using πr^2, assuming the colonies are round. Data are the mRNA +/- SE for n = 5 donor samples. The mean area of control colonies was 69.8 (+/- 42.0) and 46.9 (+/- 22.0) mm^2, for PDGF BB and AA treatments respectively (+/- SE).

i)



ii)

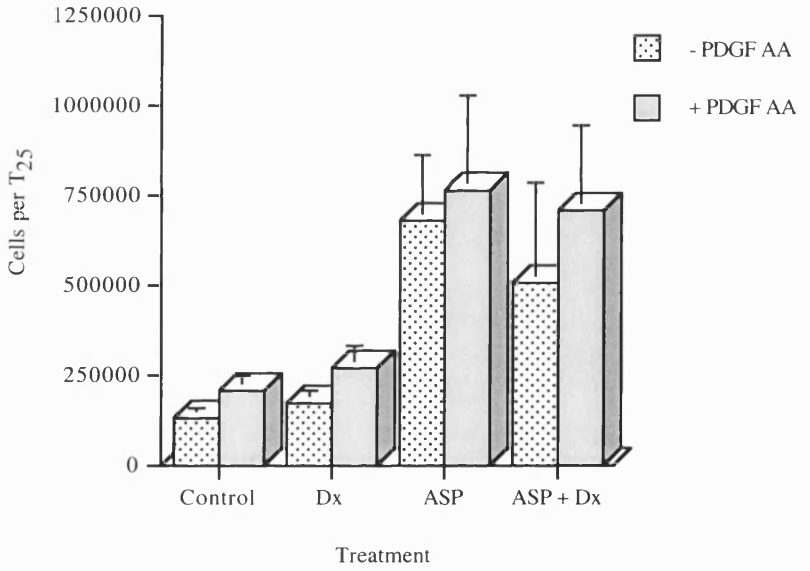


Figure 5.10 The effect of PDGF on the proliferation of CFU-F progeny

BMSC were cultured in the absence or presence of 10^{-4} M ASP and 10^{-8} M Dx and treated with 2.5 ng/ml PDGF BB (i) or AA (ii). Cells were harvested after 28 days in 1° culture by treatment with sequential trypsin-collagenase and counted electronically. Results are the mean cell number (n = 8 (i) and 6 (ii) donors) +/- SE. Control values were 78364 (+/- 27027) and 130476 (+/-16337) cells per T_{25} for BB and AA treated cultures respectively (+/- SE).

5.4 The effect of PDGF on differentiation of CFU-F progeny

5.4.1 Expression of alkaline phosphatase

With a single exception (PDGF BB + ASP) the AP activity of BMSC was increased by treatment with either PDGF homodimer (figure 5.11). A significant induction of enzyme activity resulted in the presence of ASP + Dx ($p < 0.05$). Irrespective of the absence or presence of ASP or Dx, the effect of the AA homodimer on AP expression was greater than that of the BB homodimer.

5.4.2 Mineralisation

Mineral deposition was only detected in cultures treated with ASP + Dx (visual observation, figure 5.12). PDGF BB treatment augmented this effect, but did not induce mineralisation when added to culture medium with ASP alone.

5.4.3 Surface marker expression of STRO-1 and AP

Single label experiments revealed small changes in the expression of STRO-1 and AP on BMSC when cultured in the presence of PDGF. Treatment with PDGF BB in the absence of Dx, slightly reduced the proportion of the total cell population that expressed STRO-1 (figure 5.13i) and the mean cell surface density (MFI) of STRO-1. In contrast, PDGF BB treatment in combination with Dx modestly increased the percentage of cells that expressed STRO-1, and increased the mean cell surface expression of the antigen. The proportion of cells expressing AP and the MFI were not substantially altered by PDGF BB treatment in the absence or presence of Dx (figure 5.13ii).

Dual labelling of BMSC with the monoclonal antibodies STRO-1 and B4-78 (anti-AP) consistently revealed the presence of 4 subpopulations; STRO-1^-/AP^- (R_1), STRO-1^+/AP^- (R_2), STRO-1^+/AP^+ (R_3) and STRO-1^-/AP^+ (R_4, figures 5.13iii, 5.14i, 5.14ii). Treatment with PDGF BB (figure 5.13iii) had little influence over the distribution of the cells in these populations in the absence of Dx. In the presence of Dx, treatment with PDGF BB modestly increased the proportion of cells in R_3, and reduced the proportion in R_4.

Treatment with Dx alone (figures 5.13iii, 5.14i, 5.14ii) uniformly increased the size of the STRO-1^+/AP^+ (R_3) and STRO-1^-/AP^+ (R_4) populations, whilst decreasing that of the STRO-1^+/AP^- (R_2) population. In the two donor samples investigated (figures 5.14i, 5.14ii), PDGF AA treatment did alter the distribution of cells in the four subpopulations, but the effect were inconsistent between the samples.

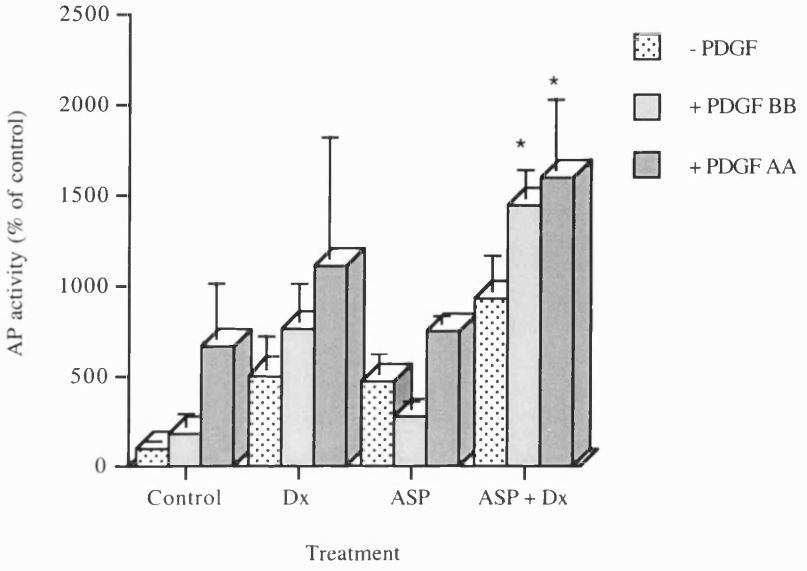


Figure 5.11 The effect of PDGF on AP biochemical activity

BMSC were cultured under standard conditions +/- 10^{-8} M Dx in the absence or presence of 2.5 ng/ml PDGF BB or AA. Cells were harvested after 28 days in primary culture, lysed and the biochemical activity of AP measured by the conversion of p-NPP to p-NP, at pH 9.2. Due to the existence of a large inter-donor variation in AP activity, the data have been presented as a % of the control value (+/-SE) for n = 3 and 4 donors for PDGF AA and BB respectively. * Indicates a significant increase in activity over ASP + Dx control culture ($p < 0.05$). The mean control value was 62.5 (+/- 22) nmol p-NP/1x10^6 cells/min.

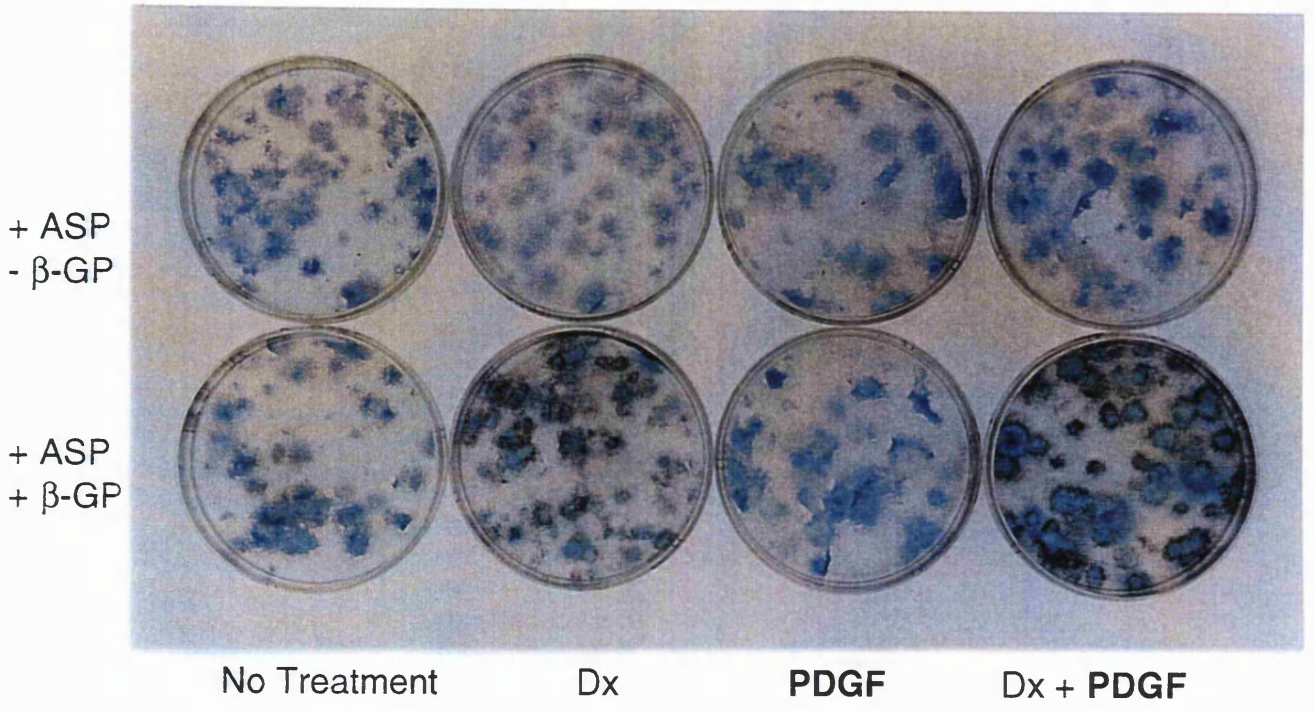
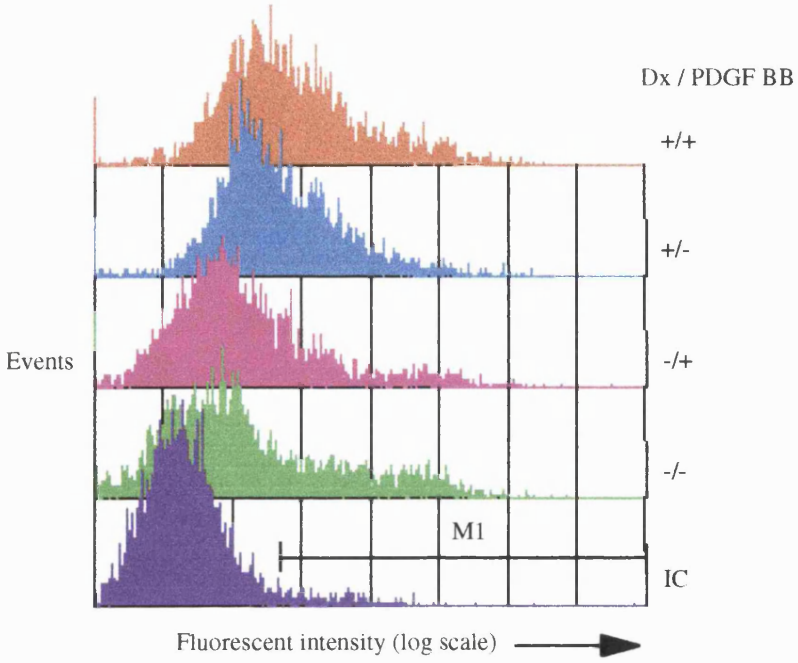


Figure 5.12 Mineralisation in cultures of BMSC treated with PDGF BB

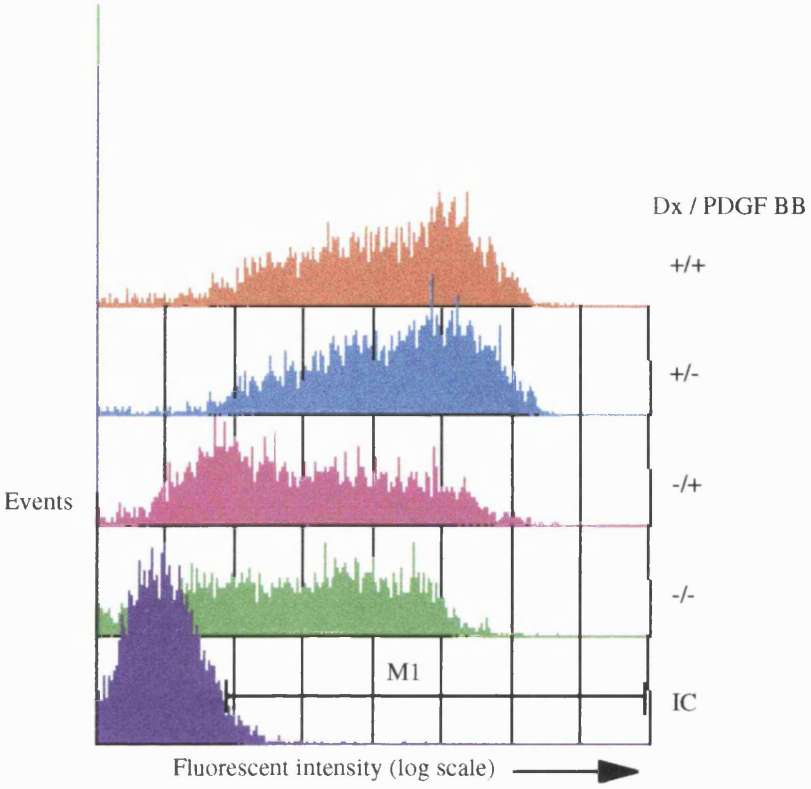
BMSC were treated with or without 10^{-8} M Dx and/or 2.5 ng/ml PDGF BB under standard 1° culture conditions for 12 days. β-glycerophosphate (β-GP) was added to half the plates for 5 days before the colonies were fixed. Cultures were fixed with NBF, stained with von Kossa stain and counter stained with methylene blue. Experiments of n = 4 donors showed similar results.



Dx/BB	Marker	% Total	Mean	MFI
IC	All	100.00	9.99	4.84
	M1	5.70	93.43	46.36
-/-	All	100.00	47.26	12.90
	M1	32.10	132.71	73.90
-/+	All	100.00	36.31	11.76
	M1	27.64	111.25	54.30
+/-	All	100.00	40.45	21.10
	M1	49.90	70.20	45.02
+/+	All	100.00	58.84	26.05
	M1	58.44	93.07	52.90

Figure 5.13i The effect of PDGF BB on BMSC expression of STRO-1

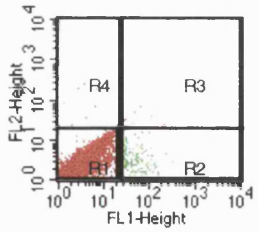
BMSC were cultured for 28 days +/- 10^{-8} M Dx in the absence or presence of 2.5 ng/ml PDGF BB. Harvested cells were dual-labelled with mAbs against STRO-1 and AP and fluorescent-conjugated 2° antibodies. Labelled cells were analysed by FACS. The results shown are representative of n = 4 donors which demonstrate similar results. The table is based on the results of the M1 marker aligned on 5% fluorescent cells in the negatively stained population. IC, negative isotype control.



Dx/BB	Marker	% Total	Mean	MFI
IC	All	100.00	3.85	3.07
	M1	4.88	15.29	12.31
-/-	All	100.00	73.09	22.77
	M1	69.06	104.27	58.82
-/+	All	100.00	97.81	28.30
	M1	72.82	132.53	58.03
+/-	All	100.00	264.64	130.42
	M1	97.12	272.37	145.89
+/+	All	100.00	231.21	103.10
	M1	93.88	246.01	129.23

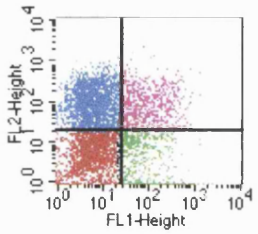
Figure 5.13ii The effect of PDGF BB on BMSC expression of AP

BMSC were cultured, harvested and stained as described in the legend of figure 5.13i. The results indicate cell surface expression of AP.



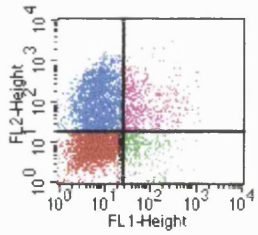
Negative isotype control

Region	Events	% Total	X Mean	Y Mean
R1	4754	95.08	5.09	3.61
R2	203	4.06	120.50	3.15
R3	16	0.32	49.48	29.59
R4	13	0.26	12.21	69.46



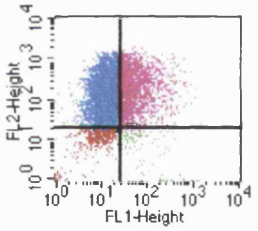
Control

Region	Events	% Total	X Mean	Y Mean
R1	1532	30.64	7.57	6.68
R2	656	13.12	168.43	4.98
R3	687	13.74	141.37	130.03
R4	2108	42.16	7.90	124.70



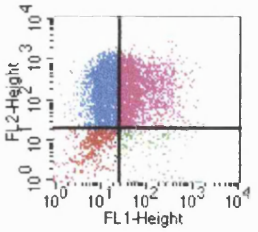
PDGF BB

Region	Events	% Total	X Mean	Y Mean
R1	1790	35.80	8.16	8.10
R2	435	8.70	171.96	6.79
R3	592	11.84	120.90	164.10
R4	2145	42.90	9.13	174.67



Dx

Region	Events	% Total	X Mean	Y Mean
R1	338	6.76	9.37	10.95
R2	117	2.34	156.79	9.47
R3	1764	35.28	81.70	320.11
R4	2792	55.84	13.21	271.33

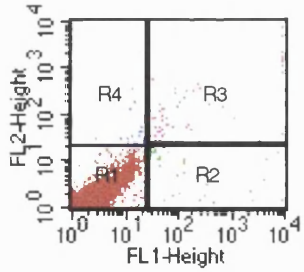


Dx + PDGF BB

Region	Events	% Total	X Mean	Y Mean
R1	457	9.14	8.93	9.16
R2	216	4.32	214.32	9.59
R3	2101	42.02	101.57	270.51
R4	2248	44.96	13.86	261.13

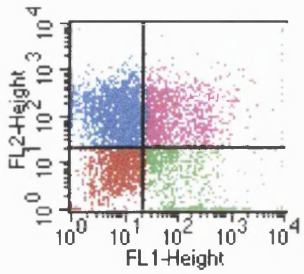
Figure 5.13iii The effect of PDGF BB on BMSC expression of STRO-1 and AP

BMSC were cultured, harvested and stained as described in the legend of figure 5.13i. The dot blots indicate the staining patterns of both antigens, where R_1 = STRO-1$^-$/AP$^-$, R_2 = STRO-1$^+$/AP$^-$, R_3 = STRO-1$^+$/AP$^+$, R_4 = STRO-1$^-$/AP$^+$.



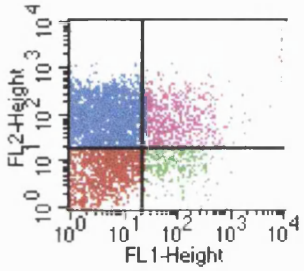
Negative isotype control

Region	Events	% Total	X Mean	Y Mean
R1	4847	96.94	3.26	2.20
R2	52	1.04	1075.06	8.35
R3	64	1.28	1562.88	493.73
R4	34	0.68	15.10	69.47



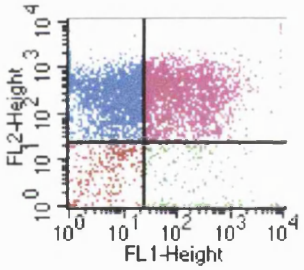
Control

Region	Events	% Total	X Mean	Y Mean
R1	1021	20.42	9.26	8.69
R2	604	12.08	302.43	7.25
R3	1116	22.32	284.44	231.79
R4	2248	44.96	9.52	168.86



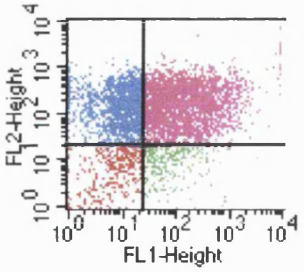
PDGF AA

Region	Events	% Total	X Mean	Y Mean
R1	1205	24.10	6.56	8.40
R2	296	5.92	123.49	10.15
R3	623	12.46	251.20	168.13
R4	2868	57.36	6.05	138.92



Dx

Region	Events	% Total	X Mean	Y Mean
R1	264	5.28	6.39	7.30
R2	147	2.94	508.12	7.22
R3	2142	42.84	264.42	363.69
R4	2440	48.80	9.51	310.85

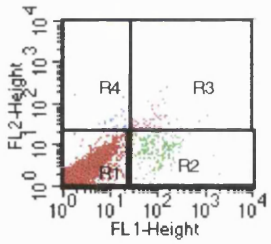


Dx + PDGF AA

Region	Events	% Total	X Mean	Y Mean
R1	360	7.20	8.39	7.91
R2	256	5.12	129.54	10.55
R3	2802	56.04	310.94	264.44
R4	1564	31.28	11.21	218.69

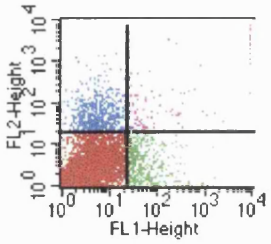
Figure 5.14i ***The effect of PDGF AA on BMSC expression of STRO-1 and AP: donor 1***

BMSC were cultured, harvested and stained as described in the legend of figure 5.13i with 2.5 ng/ml PDGF AA. The dot blots indicate the staining patterns of both antigens, where R_1 = STRO-1$^-$/AP$^-$, R_2 = STRO-1$^+$/AP$^-$, R_3 = STRO-1$^+$/AP$^+$, R_4 = STRO-1$^-$/AP$^+$.



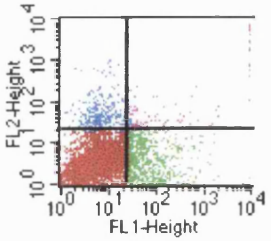
Negative isotype control

Region	Events	% Total	X Mean	Y Mean
R1	4696	93.92	3.81	3.04
R2	205	4.10	118.76	8.83
R3	72	1.44	551.20	268.78
R4	33	0.66	15.97	40.85



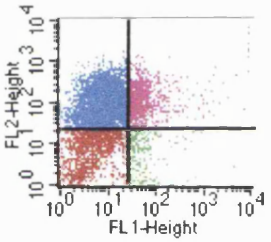
Control

Region	Events	% Total	X Mean	Y Mean
R1	3931	78.62	5.68	3.93
R2	416	8.32	95.82	5.25
R3	96	1.92	1494.73	668.13
R4	568	11.36	7.64	100.71



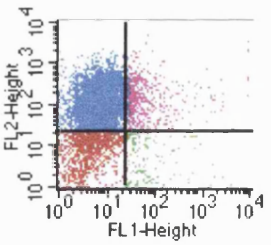
PDGF AA

Region	Events	% Total	X Mean	Y Mean
R1	3968	79.36	7.27	4.32
R2	641	12.82	199.16	5.61
R3	103	2.06	1495.45	556.13
R4	295	5.90	9.48	86.38



Dx

Region	Events	% Total	X Mean	Y Mean
R1	1009	20.18	6.17	9.17
R2	197	3.94	103.21	8.24
R3	755	15.10	210.66	193.08
R4	3058	61.16	10.57	133.49



PDGF AA + Dx

Region	Events	% Total	X Mean	Y Mean
R1	934	18.68	5.96	9.70
R2	146	2.92	257.79	7.53
R3	435	8.70	265.51	241.08
R4	3502	70.04	8.68	170.62

Figure 5.14ii The effect of PDGF AA on BMSC expression of STRO-1 and AP: donor 2

BMSC were cultured, harvested and stained as described in the legend of figure 5.13i with 2.5 ng/ml PDGF AA.

5.4.4 Expression of bone marker mRNA

The expression of selected 'osteoblastic' mRNAs was assessed using RT-PCR. Complementary DNA encoding bone sialoprotein, osteocalcin, oestrogen receptor, and parathyroid hormone receptor was amplified from mRNA extracts with specific oligonucleotide primers. Using this method of analysis, which at best is semiquantitative, there was no consistent effect of any of the treatment regimens on the expression of transcripts for the parathyroid hormone receptor, and oestrogen receptor (figures 5.15i and 5.15ii). Treatment with Dx, and albeit to a lesser extent, PDGF BB increased the expression of BSP transcripts in cultures derived from 3 of 4 donors. When added in combination the effect on BSP transcript expression was intermediate between that of Dx and PDGF alone. In cultures derived from 3 of 4 donors PDGF BB treatment, irrespective of the absence or presence of Dx, slightly decreased osteocalcin transcript expression.

5.4.5 Osteocalcin synthesis

Secretion of 1,25-dihydroxyvitamin D_3-induced osteocalcin was measured in the culture medium of two experiments using cells from separate donors, one treated with PDGF BB, the other with PDGF AA. PDGF BB reduced the amount of osteocalcin secreted by BMSC -/+ Dx, but no consistent effect of PDGF AA treatment was observed (figure 5.16).

5.5 Interaction of PDGF with IGF-I responsive pathways

PDGF BB increased proliferation measured as the incorporation of [^{3}H]-thymidine in the absence or presence of Dx (figure 5.17). In contrast, IGF-I treatment increased [^{3}H]-thymidine incorporation only in the presence of Dx, and its effect was not clearly dose dependent. Irrespective of the absence or presence of Dx, the effect of both agents added in combination was no different to that observed in the presence of PDGF BB alone.

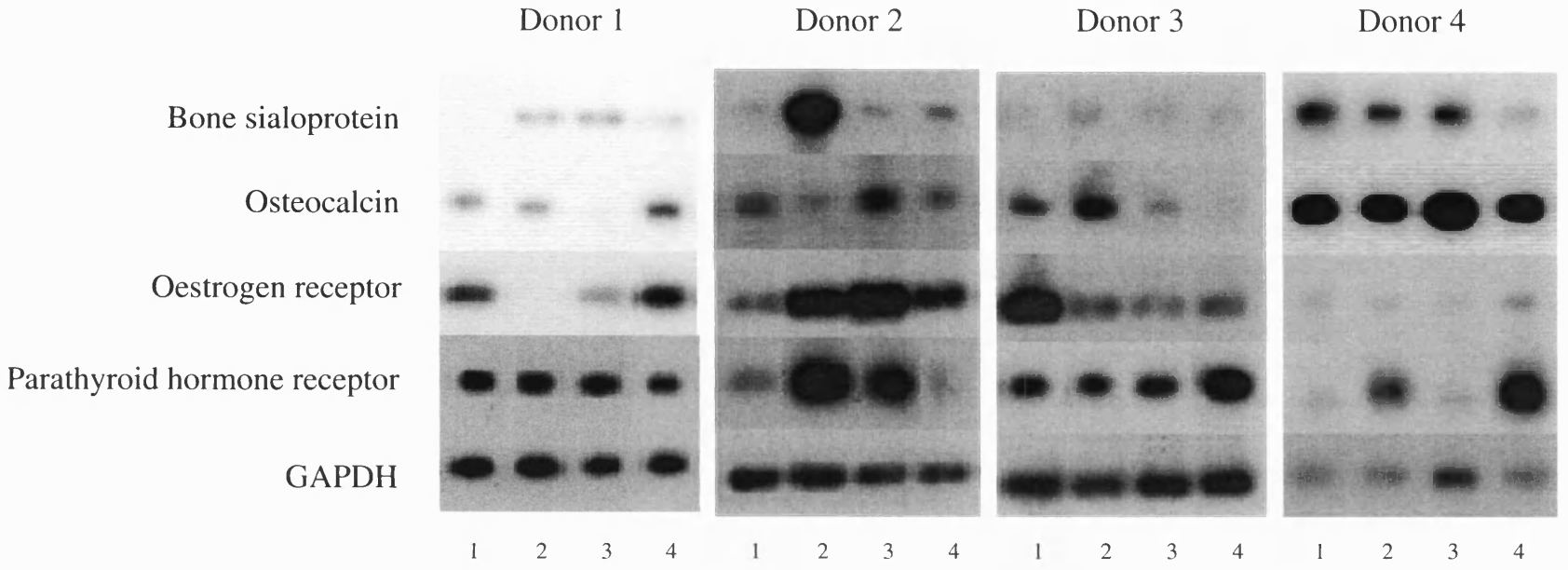


Figure 5.15i The effect of PDGF BB on osteoblast marker mRNA expression in BMSC

cDNA prepared from BMSC cultured in medium alone (1) or medium + 10^{-8} M Dx (2), 2.5 ng/ml PDGF BB (3) or Dx and PDGF in combination (4). PCR was performed using primers for the indicated osteoblast markers and the production separated by agarose gel electrophoresis. Southern blots were then prepared and hybridised with radiolabelled probes complementary to sequences internal to those of each primer pair. Digitised images of the resulting autoradiographs were captured using the ImageDok programme and analysed using Phoretix ID software package.

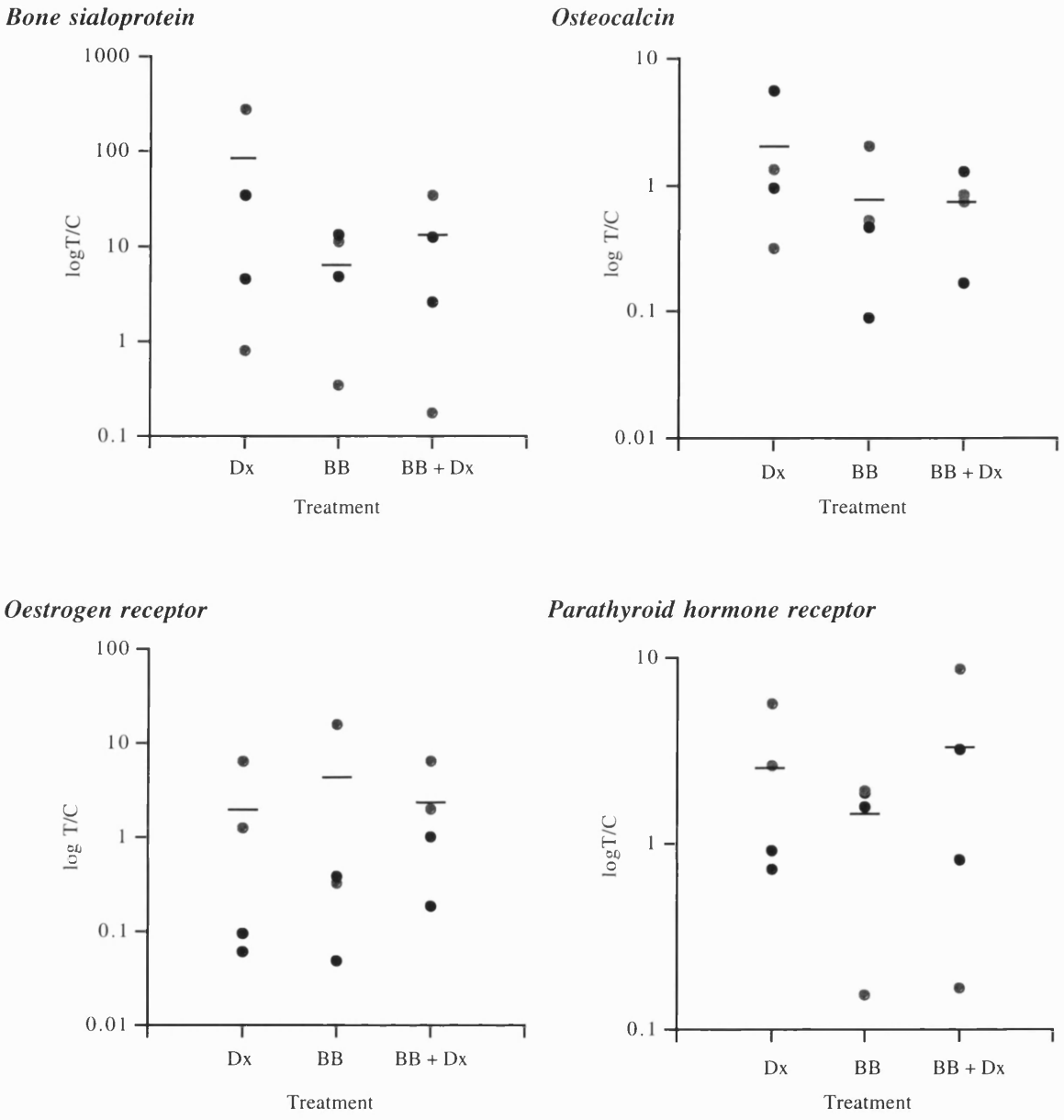
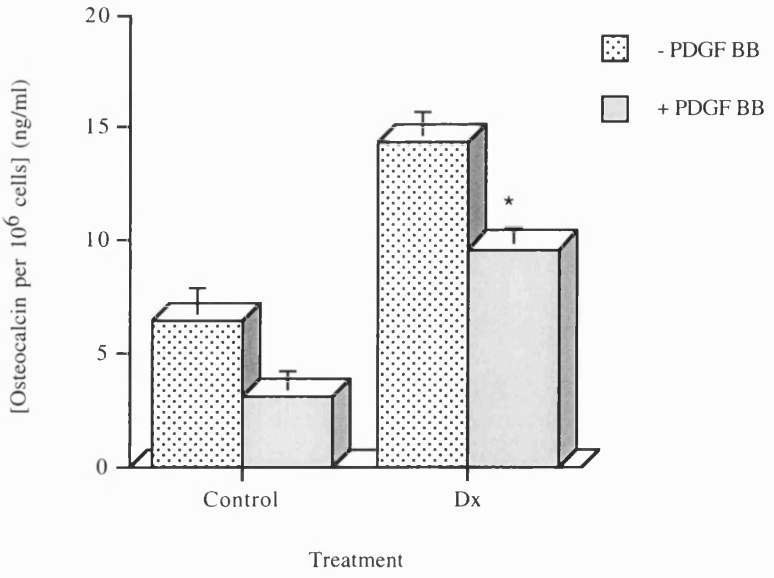


Figure 5.15ii The effect of PDGF BB on expression of osteoblast mRNAs in 1° cultures of BMSC: Results of image analysis

The data from images in 5.15i have been corrected for differences in the amount of input cDNA using the GAPDH product as an internal standard and expressed as a T/C ratio. Horizontal bars indicate the mean of n = 4 donors.

i)



ii)

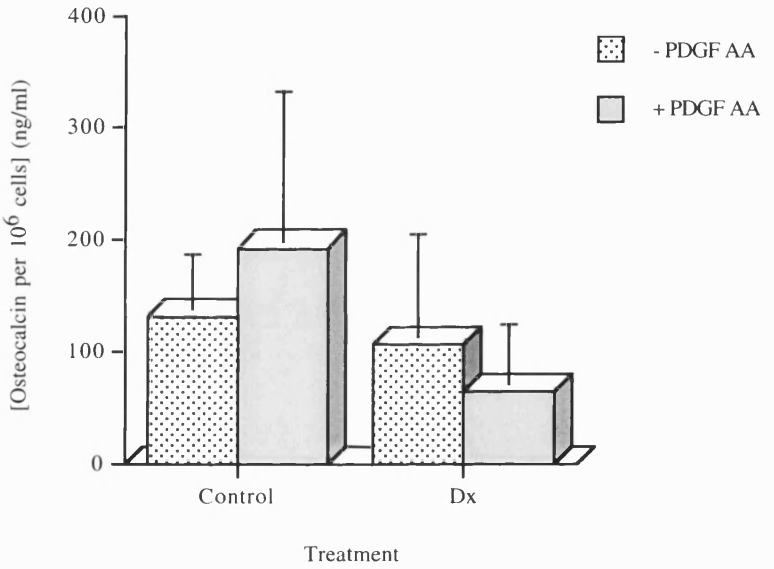


Figure 5.16 The effect of PDGF on 1,25-dihydroxyvitamin D_3-induced secretion of osteocalcin

BMSC were cultured under standard 1° conditions for 20 days (+/- Dx) in the absence or presence of 2.5 ng/ml (i) PDGF BB or (ii) AA. To induce the secretion of osteocalcin, the cultures were additionally supplemented with 1,25-dihydroxyvitamin D_3 for the final 48 hours. Osteocalcin secreted into the medium was measured using a commercially available ELISA kit (Novocalcin, Metra Biosystems Inc. section 2.6.5). Results are the mean of n = 4 +/- SE, and * indicates significant ($p < 0.05$) inhibition compared to ASP + Dx control treatment.

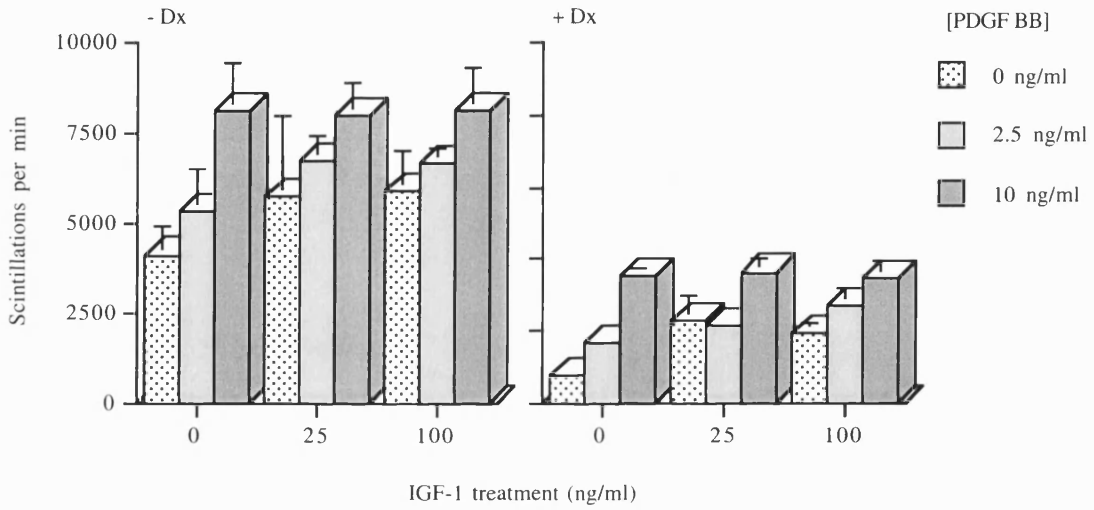


Figure 5.17 The effect of IGF-I combined with PDGF BB on BMSC proliferation

Secondary BMSC were seeded into 96 well plates at $1x10^4$ cells per cm^2 and treated with IGF-I (25 or 100 ng/ml) in the absence or presence of PDGF BB (2.5 or 10 ng/ml). The experiments were performed in medium + 10% FCS +/- 10^{-8} M Dx. Cultures were treated for 48 hours and then pulsed with [^{3}H]-thymidine for 24 hours. [^{3}H]-thymidine incorporation was measured on a β-counter. The results shown are the mean +/- SE for n = 3 donors.

5.6 Discussion

For therapies involving reimplantation of *ex-vivo* expanded stromal cells, progenitor cells are often more appropriate for treatments in which restoration of a functional cell population is necessary. Local growth factors can then influence the commitment of the cells and restore widespread cell activity. Terminally differentiated cells have a limited lifespan and are non-proliferative, and thus are only biologically active on a short term basis. In this investigation the ability of PDGF to promote the *ex-vivo* expansion of human bone marrow stromal cells without altering their potential for osteogenic differentiation was investigated.

Initial characterisation studies determined that PDGF A and B chains were synthesised by human bone-derived cells. α and β receptor subunits were also found to be expressed on ~40-60% of the cultured cell population. The size of the PDGF receptor chains determined by PAGE was consistent with the reported sizes of 180 kDa (PDGFRβ, Bowen-Pope *et al*, 1985) and 200 kDa (PDGFRα, Centrella *et al*, 1992). Expression of the PDGF A and B isoforms and the presence of α and β receptors supports the role of PDGF as important autocrine and/or paracrine regulators of BMSC proliferation, and possibly differentiation.

With Dx treatment, RT-PCR suggests that expression of the PDGF B chain is decreased, whereas expression of the PDGF A chain is increased. It would be necessary to confirm this using Northern analysis, and direct measurement of PDGF A and B chain proteins by ELISA with a greater sample number. Flow cytometry data indicates that the proportion of the BMSC population that express PDGFRα and PDGFRβ was also decreased with Dx treatment. As Dx is a known inducer of osteogenic differentiation these responses suggest that more mature cells of the osteoblast lineage have reduced expression of PDGF B or receptor peptides. As PDGF is reported to induce a mitogenic response in mesenchymal cells, this may present a rationale for the decrease in proliferation observed in Dx treated osteoblastic cells. This suggests, therefore, that in normal unstimulated cells, Dx treatment could alter the PDGF mediated autocrine or paracrine pathways. Also these cultures represent a heterogeneous population of cells of multiple mesenchymal stromal lineages and it is not clear whether receptors are expressed exclusively on the cells committed to the osteoblastic lineage, and if so at what stage(s) during their differentiation. A multiparameter flow cytometric analysis using fluorescently labelled antibodies recognising STRO-1, AP and PDGF receptors might be informative in this regard. It is known that the proliferative potential of cells of the osteoblastic lineage in inversely related to their extent of differentiation (Stein *et al*, 1990). Whether down regulation of the receptors and PDGF B chain is a direct result of Dx treatment or an increased maturity of the cells remains to be determined. The responsiveness of more immature cells to PDGF is supported by Hsieh and Graves (1998) who found that PDGF BB stimulated DNA synthesis in cultures of foetal rat calvarial cells soon after explant, but this diminished as the cells matured. Yu *et al* (1997)

also showed that expression of PDGF receptor subtypes on rat foetal calvarial cells reached a maximum coincident with the upregulation of AP expression, but then declined as the cells underwent further maturation.

PDGF increased the number of cells in cultures of BMSC. This was the result of increased proliferation demonstrated by an increase in cell number and colony size, rather than in CFE. These results are consistent with PDGF acting in the initial stages of culture to increase the size of the adherent cell population (Herbertson and Aubin, 1995a, Yu *et al*, 1997, Hsieh *et al*, 1998), and concordant with the finding of Gronthos and Simmons (1995) who demonstrated an increase in the size, but not the total number of colonies formed in PDGF supplemented serum-free medium when compared with serum-replete conditions. PDGF is known to be an essential requirement for mesenchymal cell proliferation *in vitro* (Graves *et al*, 1989) and it is now well documented that PDGF is required in the early stages of colony formation. Recent studies investigating factors that regulate growth and development of primitive bone marrow precursors have recently defined that clonogenic growth of CFU-F can be supported under serum-deprived conditions, in the presence of ASP and Dx, but is absolutely dependent on an exogenous source of growth factor, with PDGF and epidermal growth factor (EGF) demonstrating the greatest ability to support growth (McIntyre and Bjornson, 1986, Gronthos and Simmons, 1995). In a recent study, Kuznetsov *et al* (1997) demonstrated that only anti-PDGF antibodies specifically suppressed colony formation, when neutralising antibodies against several growth factors were used.

In this study, PDGF was found to limit the number of clonogenic cells that became committed to the osteogenic lineage shown by a reduction in the proportion of AP positive colonies. The PDGF isoforms were found to differ in their functional properties, with PDGF AA having a greater effect on osteogenic differentiation than PDGF BB, based on expression of AP. However, markers of osteoblastic differentiation did not reveal any specific induction of osteogenic differentiation by PDGF BB. Flow cytometry using mAbs recognising the STRO-1 antigen and BLK isoenzyme of AP sub-divides the human BMSC cultures into four distinct populations representing cells at different stages of osteogenic differentiation (section 5.4.3, figure 5.18). A working hypothesis has been assigned that the cells within these populations are functionally distinct and form part of a lineage progression: multipotential precursors being present in the STRO-1^+/AP^- population, committed osteoprogenitors in the STRO-1^+/AP^+ population, and maturing osteoblasts in the STRO-1^-/AP^+ population. The STRO-1^-/AP^- population is thought to contain cells that are not of the osteoblastic lineage. When analysed by flow cytometry, treatment of BMSC with PDGF BB, even when added in combination with Dx and/or ASP did not significantly alter the distribution of cells within these populations, indicating that the mitogenic effects of this growth factor are non-selective and not coupled specifically to the proliferation of cells of the osteoblast lineage.

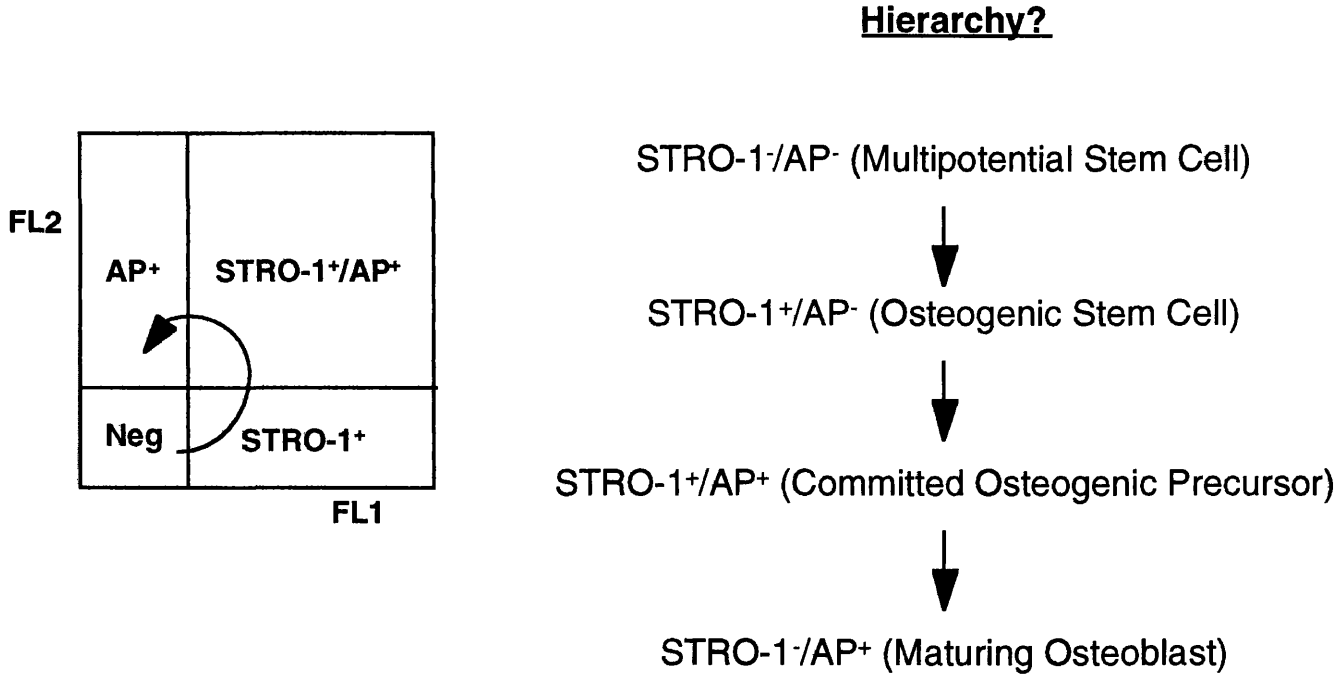


Figure 5.18 Analysis of human marrow stromal cells by flow cytometry using STRO-1 and B4-78 (α-AP) mAbs

Certainly neither isoform appeared to substantially alter the ability of Dx to increase the proportion of cells present in the STRO-1+/AP$^+$ and STRO-1-/AP$^+$ populations. This is in marked contrast to that of basic fibroblast growth factor (FGF-2), which in addition to being a potent mitogen also promotes osteogenic differentiation in this cell culture system (Walsh and Beresford, 1995).

Analysis of the effects of PDGF BB on the expression of AP in this cell culture system, revealed that a modest increase in AP activity observed in PDGF-treated cultures could not be accounted for by an increase in the mean fluorescent index. This suggests that PDGF BB exerts its small effect on the expression of AP by modulating the catalytic activity of pre-existing enzyme in this culture system. This may otherwise be attributed to the increase in proliferation of cells expressing AP. Further evidence based on the deposition of mineral and expression of markers of the osteoblast lineage (parathyroid hormone, osteocalcin, oestrogen and BSP) demonstrated that PDGF BB did not substantially alter the differentiation of these cells, and acted to moderate the effects of Dx on osteogenic differentiation. A decrease in osteocalcin mRNA and peptide expression compared to control cultures demonstrated the maintenance of an immature osteoblastic population in PDGF BB treated cultures.

Attempts to show the interaction of PDGF with IGF-I did not reveal any further mitogenic effect of the growth factors in combination above that of PDGF alone. The lack of a response may have been due primarily to one of the doses of IGF-I or PDGF being too high, thus producing a maximum response alone and masking a dual effect. IGF-I is abundantly produced by cells of the osteoblastic lineage which might also have masked the effect of exogenously added growth factor. This was addressed by attempting to block the IGF-I autocrine loop using an anti type I IGF receptor antibody (αIR-3, Kappel *et al*, 1994), and antisense oligo-deoxynucleotides directed against type I receptor mRNA. However, these experiments did not lead to any alteration in cell proliferation even when the experimental design was varied and various methods of evaluating viable cell numbers assessed ([^{3}H]-thymidine incorporation, MTT (Mossmann, 1983), neutral red (Lowik *et al*, 1993) and cell counts).

In conclusion, the major effect of PDGF BB in this culture system, was therefore mitogenic, acting to promote *ex-vivo* expansion of human BMSC without altering the potential for osteogenic differentiation. The results suggest that the actions of the two isoforms in this cell culture system are distinct and although both isoforms of PDGF induced proliferative response in human marrow stromal cells at all stages of osteoblast maturity in this system, PDGF AA moderately promoted osteoblast maturation. Overall, these results are supported by Cassiede *et al* (1996) and Yu *et al* (1997) who found PDGF to be mitogenic for less differentiated cells of the osteoblastic lineage. In rat marrow stromal cultures Tanaka and

Liang (1995) found PDGF BB treatment alone increased expression of osteopontin mRNA, with no effect on expression of collagen type I or AP. Addition of PDGF to Dx treated cells reduced collagen type I and AP expression in this rat model and under both conditions, expression of osteocalcin was inhibited. Furthermore, in foetal rat bone, AP activity was reduced with PDGF treatment (Herbertson and Aubin, 1992, Cassiede *et al*, 1996, Hsieh and Graves, 1998), with PDGF BB having a greater effect than AA (Centrella *et al,* 1991). Thus expression of markers of osteoblastic differentiation support evidence that PDGF BB maintains an early osteoprogenitor population. However, in contrast to these results, it has recently been found that pulse treatment of PDGF induced mineralisation in contrast to continuous treatment which inhibits differentiation (Hsieh and Graves, 1998).

PDGF is thus a candidate mitogen for the *ex-vivo* expansion of human bone marrow stromal cells that retain an immature phenotype, but possess osteogenic potential, and might be used for tissue reconstruction and the healing of bone defects. To evaluate whether these cells are able to produce bone *in vivo,* and to fully establish the effects of PDGF on the osteogenic capacity of the cells in the absence or presence of Dx, it would be of value to test these populations in an *in vivo* assay of osteogenesis, by implanting PDGF treated cells into diffusion chambers, or into open-transplant assay with incorporation of a nuclear marker as in experiments of Krebsbach *et al* (1997).

Chapter 6

An investigation of the effects of ageing and gender on BMSC

6.1 Introduction

Osteoporosis is a disease which is manifested as a result of the combination of a large number of factors. These are based on the age of onset, sex, incidence pattern, distribution of bone loss, and the effects of a decline and/or loss of gonadal steroids on a predisposing factor. A progressive decline in bone mass and strength which occurs as a consequence of the ageing process leads to idiopathic (age related), type I osteoporosis. Post-menopausal osteoporosis (type II) is characterised by a rapid phase of bone loss associated with a decrease in circulating oestrogen at menopause. Later on men and women lose bone at similar rates (Riggs and Melton, 1986). At the tissue level, bone loss is due to an increase in the number and activity of osteoclasts with a possible prolongation of their functional lifespan (Riggs *et al*, 1998). This afflicts cortical and trabecular bone to a similar extent. Age-related changes in osteoblast function may play an important role in further bone loss, and a gradual decline in thickness of the remaining trabeculae is postulated to be due to a decline in the osteoblast number relative to the amount of bone formation required, rather than a change in osteoblast activity. Since osteoblasts are the primary effectors of bone formation *in vivo*, age-related changes in osteoprogenitor number may have an important effect on the production of osteoblasts. In adults, osteoblasts are derived from clonogenic precursors CFU-F, which are associated with the soft fibrous tissue of the marrow stroma (Beresford, 1989). It is postulated that a deficit in osteoblast numbers could be due to a secondary consequential decline in the CFU-F population with age.

Studies in experimental animals (Tsuji *et al,* 1990, Egrise *et al,* 1992, Liang *et al*, 1992, Jilka *et al*, 1996) have yielded results consistent with the hypothesis that there is an age-related decline in bone marrow CFU-F. The data from human studies have been less unequivocal with evidence for and against a decline in osteoblasts and precursor cells having been reported (Morike *et al,* 1993, Shigeno and Ashton, 1995, Glowaki, 1995, Cheng *et al*, 1996, dePollak *et al*, 1997, Oreffo *et al,* 1998).

Hence, the purpose of this investigation was to determine whether the size of the CFU-F population in human marrow is related to donor age, as a total population or in donors aged above 50 years, and whether it is associated with gender. To establish whether such associations exist, we analysed the colony forming efficiency and proliferative capacity of marrow stromal cells in primary culture, in a study of 56 human patients (table 6.1). The prevalence of osteogenic precursors was estimated by counting AP positive colonies. Secondly, this study also investigated the influence of gender on colony formation and proliferation, irrespective of the effects of donor age. The effect of treatment with Dx was also evaluated.

6.1.1 Statistical analysis

The effect of age on growth parameters of CFU-F was assessed using Spearmans rank correlation coefficient (SRCC, StatView v4.51). Data is shown as bivariate scatterplots, with regression lines of which the slope is indicated by R^2, the lines either side being the 95% confidence limits of the mean. The size of the cohort was not large enough to allow multivariate analysis, so a subset analysis was performed in an attempt to examine the possible effects of gender and Dx treatment while controlling for any effects of age. Results of BMSC colony formation, and proliferation, arising under culture conditions in the absence or presence of Dx were grouped, and comparisons between treatments and gender were made using the Mann-Whitney U test. Statistical tests are described in detail in the section 2.7. Results of the analyses correlating to figures in this section are shown in appendix II.

6.2 Results

BMSC from 56 human donors were cultured under standard primary conditions (section 2.2.1.1) in the absence or presence of Dx. The ages of these patients are shown in figure 6.1.

		Total donors			Donors aged ≥ 50 years		
		Male	Female	Both	Male	Female	Both
CFE	**Age range**	15 - 84	36 - 84	15 - 84	50 - 84	60 - 84	50 - 84
	Mean age	60.5	64.5	61.8	65.5	70	66.9
	(n)	34	22	56	24	14	38
Proliferation	**Age range**	19 - 85	26 - 80		50 - 85	57 - 80	
	Mean age	57.9	58.4	58.1	64.5	70.6	66.2
	(n)	20	10	30	16	7	23

Table 6.1 Summary statistics for the donors used in this study

6.2.1 CFE

6.2.1.1 The effect of age on CFE

In the cohort studied, there was no evidence for an effect of donor age on colony formation (total or AP positive) irrespective of the absence or presence of Dx (figure 6.2).

6.2.1.2 The influence of gender

Analysis of the data for male and female donors separately did not reveal the existence of a gender specific effect of ageing on colony formation, either in the absence or presence of Dx (figures 6.3 and 6.4).

6.2.1.3 The influence of gender in donors ≥ 50 years of age

Due to evidence for decreased bone mass with an increase in age, particularly in post-menopausal women, the colony formation arising from marrow of donors older than 50 years was analysed independently (figures 6.3 and 6.4). The analysis did not reveal any relationship between total or AP positive colony formation and the age of male or female donors, either in the absence or presence of Dx.

6.2.2 Proliferation

6.2.2.1 The effect of age on proliferation

The influence of donor age on the proliferation of BMSC in primary culture was investigated in the absence or presence of Dx. In both cases, the number of cells harvested at the end of primary culture was independent of the age of the donor (figure 6.5).

6.2.2.2 The influence of gender

Analysis of the data for male and female donors separately did not reveal the existence of a gender specific effect of ageing on cell number, either in the absence or presence of Dx (figure 6.6).

6.2.2.3 The influence of gender in donors ≥ 50 years of age

Analysis of the data for patients ≥ 50 years split by gender did not reveal the existence of a statistically significant association between ageing and proliferation in cultures established from either male or female donors (figure 6.6).

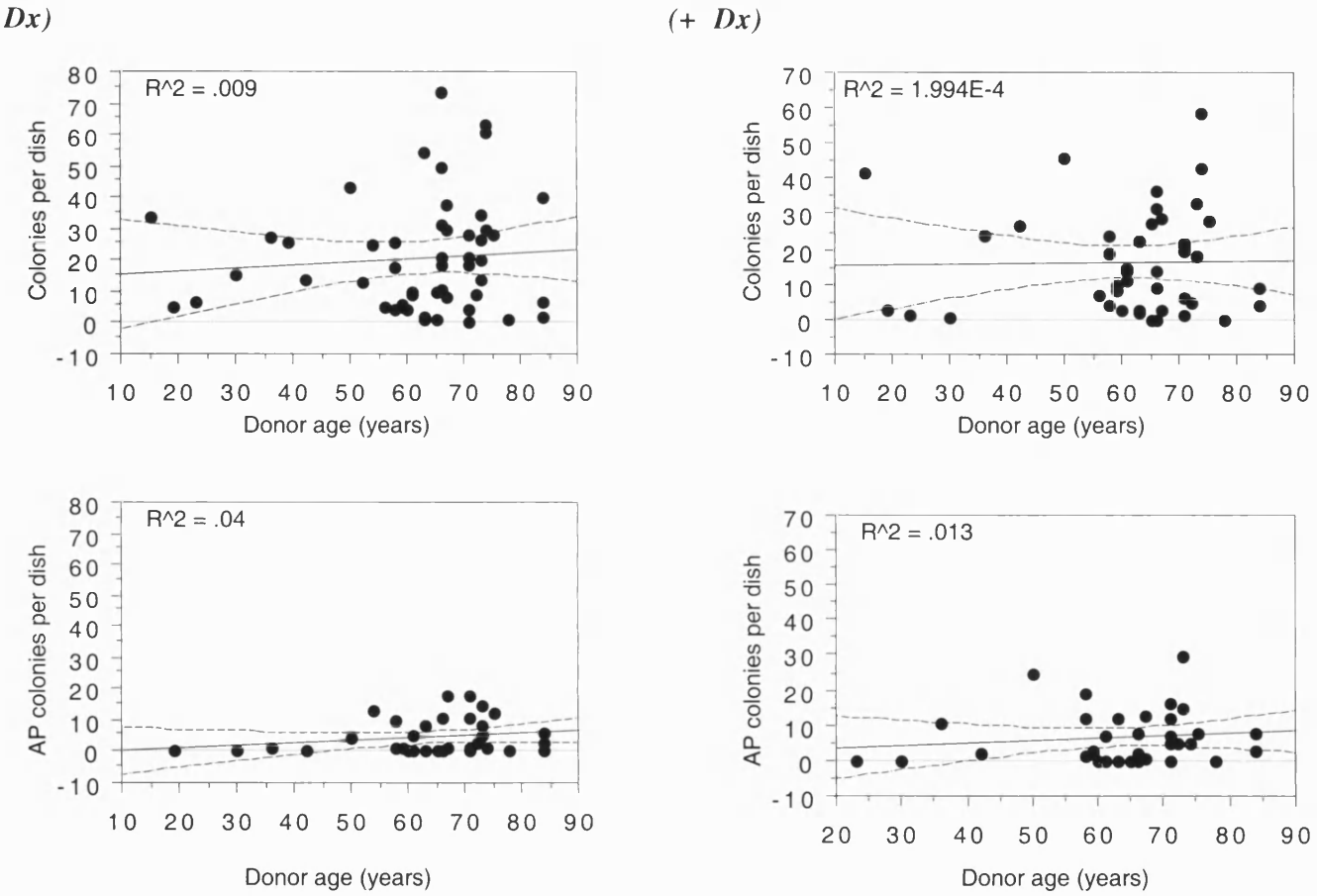


Figure 6.2 Influence of donor age on total and AP+ colony formation

BMSC were cultured for 18 days under standard 1° conditions in the absence or presence of 10^{-8} M Dx. Colonies were then fixed, and stained with Fast-red TR and methylene blue. The scattergrams show CFE plotted against the age of the donor from whom the marrow sample was taken. R^2 indicates the slope of the regression line which is plotted with 95% confidence limits.

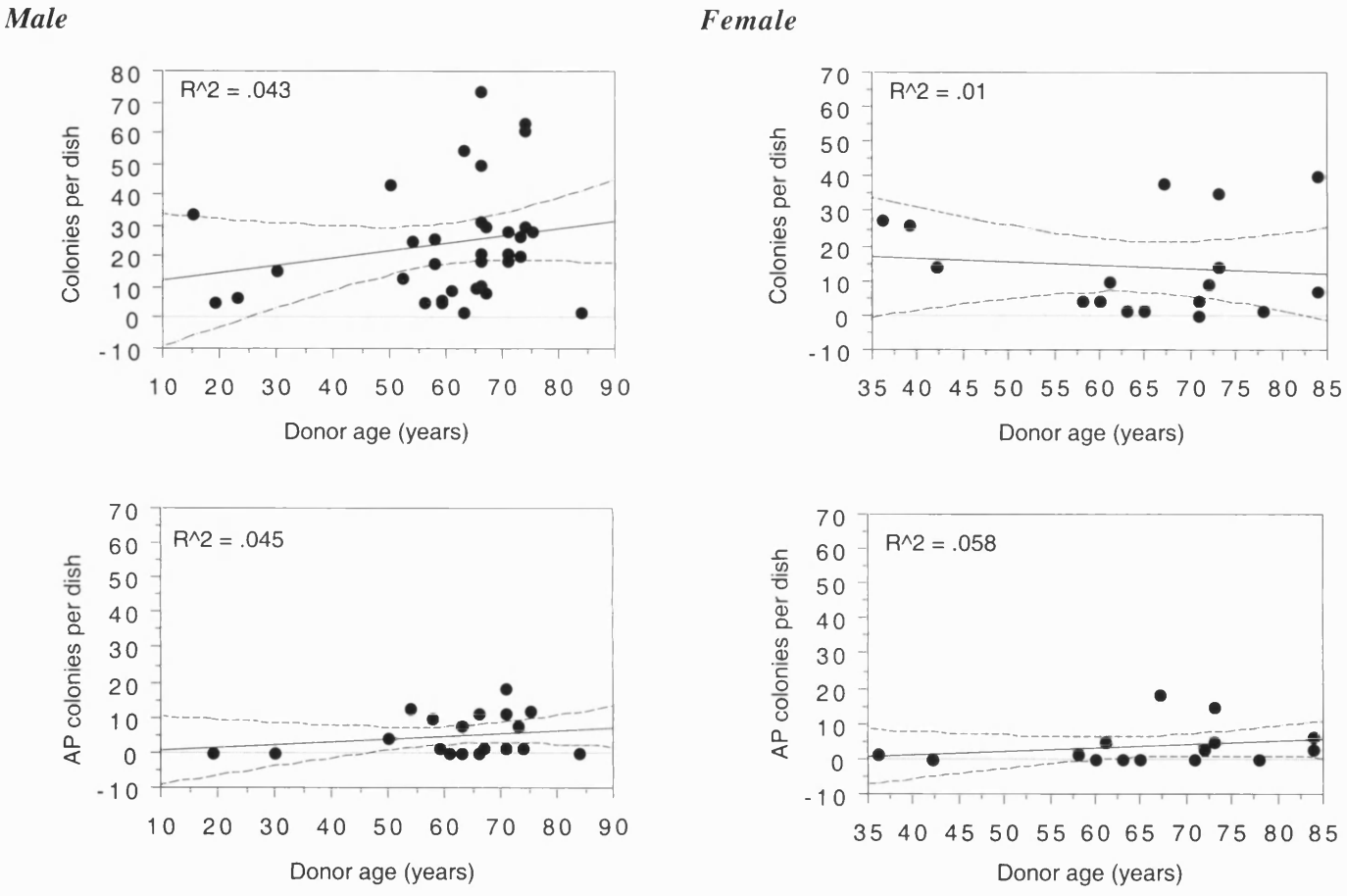


Figure 6.3 Influence of male and female donor age on total and AP+ colony formation

BMSC were cultured for 18 days under standard 1° conditions in the absence of Dx. The results in figure 6.2 were divided according to donor gender.

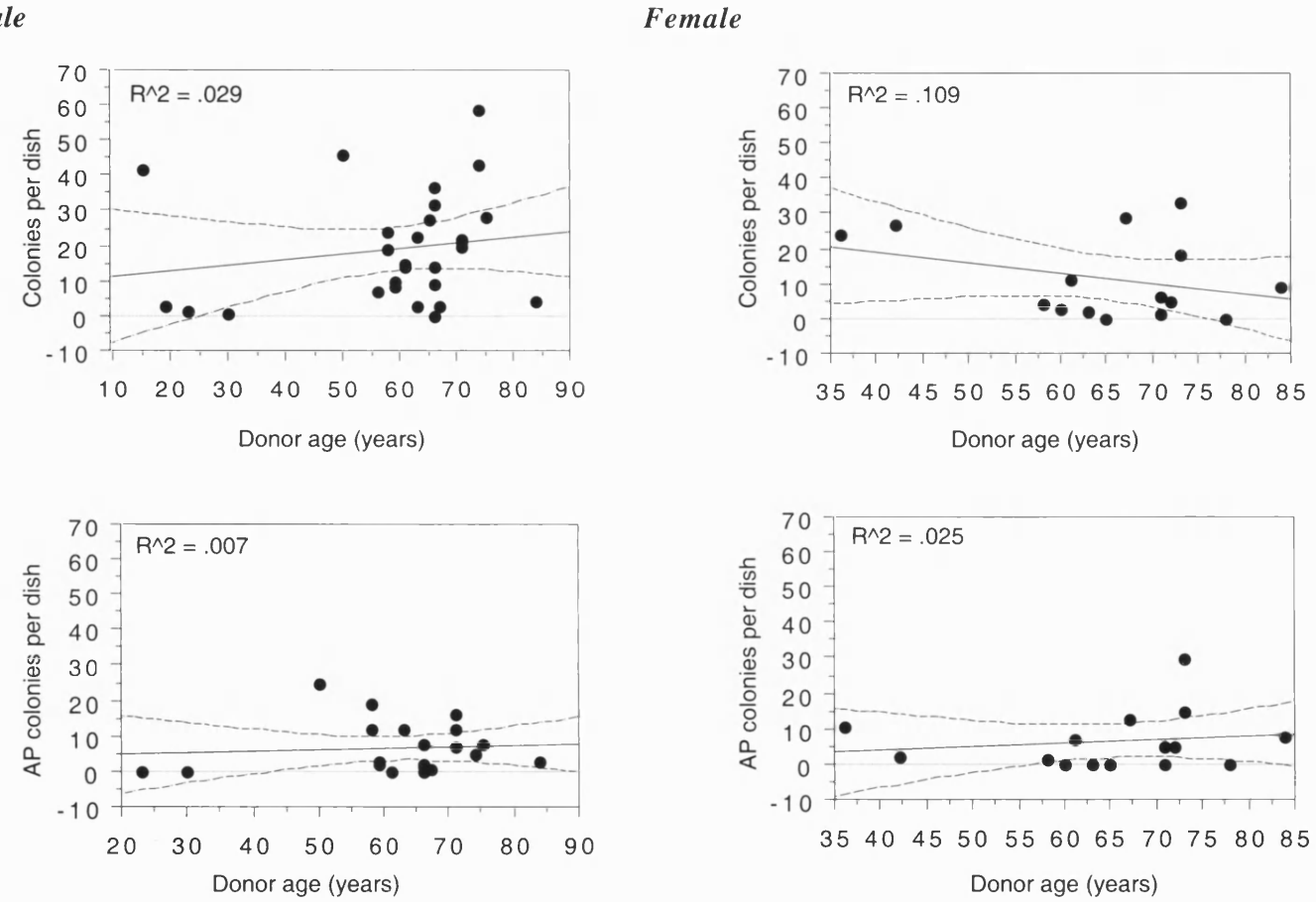


Figure 6.4 Influence of male and female donor age on total and AP+ colony formation

BMSC were cultured as described in figure 6.2 in the presence of Dx. The results shown in figure 6.2 were divided according to the gender of the donor.

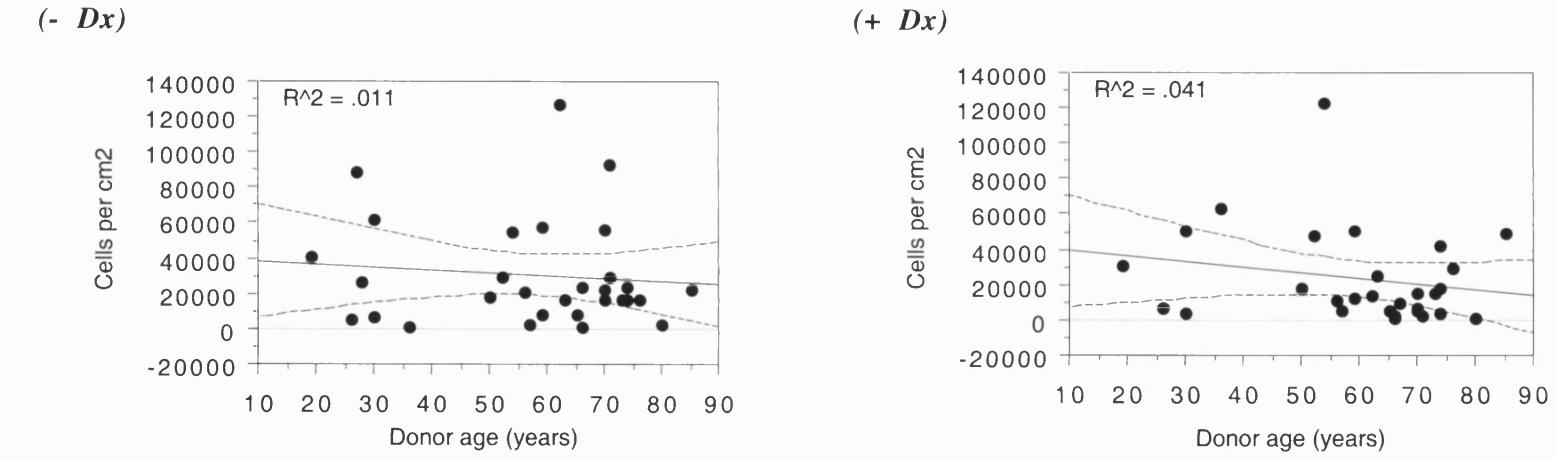


Figure 6.5 Influence of donor age on proliferation

BMSC were cultured for 18 days under standard 1° conditions in the absence or presence of Dx. Cells were harvested by sequential collagenase-trypsin digestion and counted electronically. The scattergrams show the cell count plotted against the age of the donor from whom the marrow sample was taken. R^2 indicates the slope of the regression line which is plotted with 95% confidence limits.

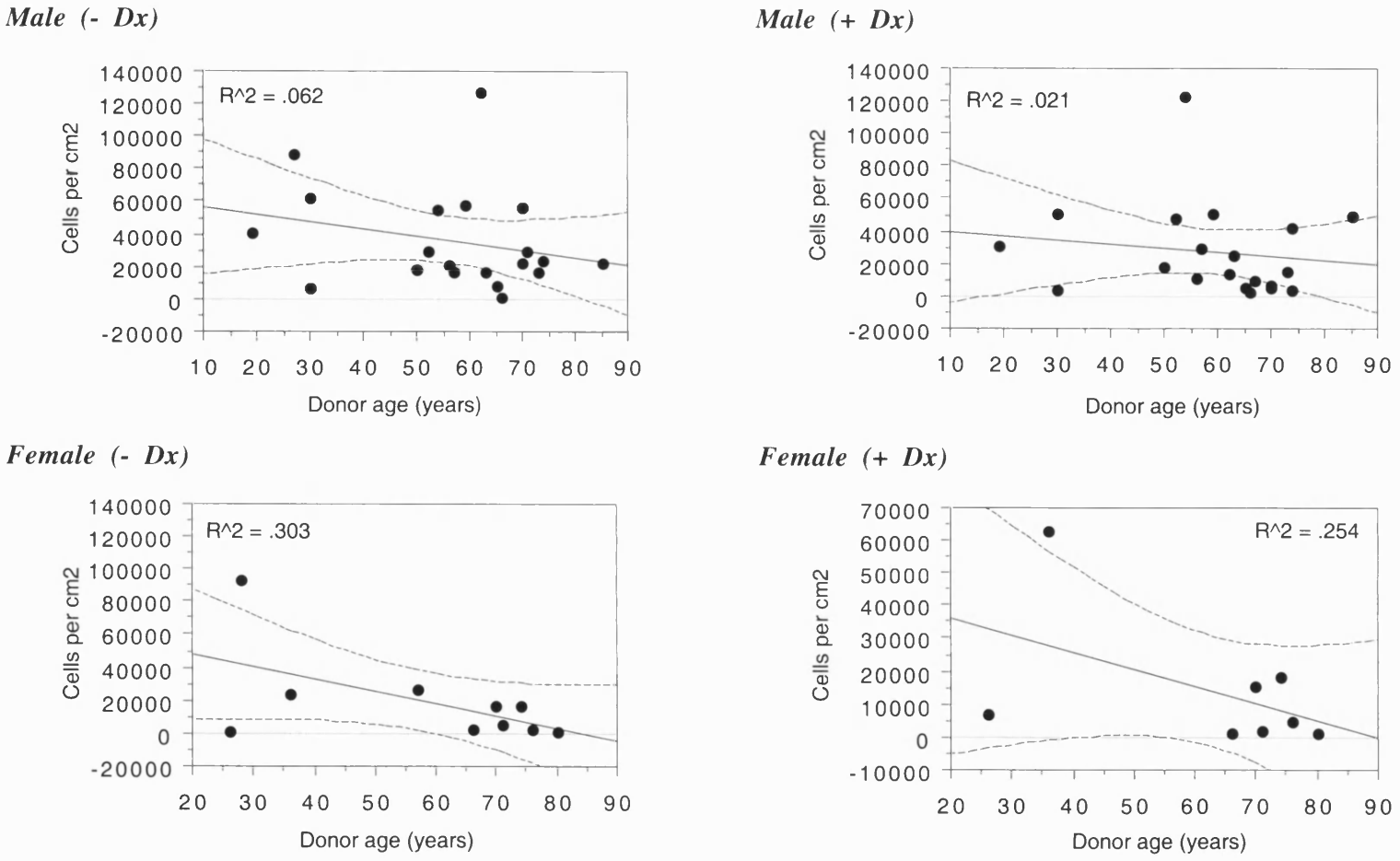


Figure 6.6 Influence of male and female donor age on cell proliferation

BMSC were cultured for 28 days under standard 1° conditions in the absence or presence of Dx. Cells were harvested by sequential collagenase-trypsin digestion and counted electronically. The results described in figure 6.5 were divided according to the gender of the donor.

6.3 Comparisons between genders and treatment irrespective of age

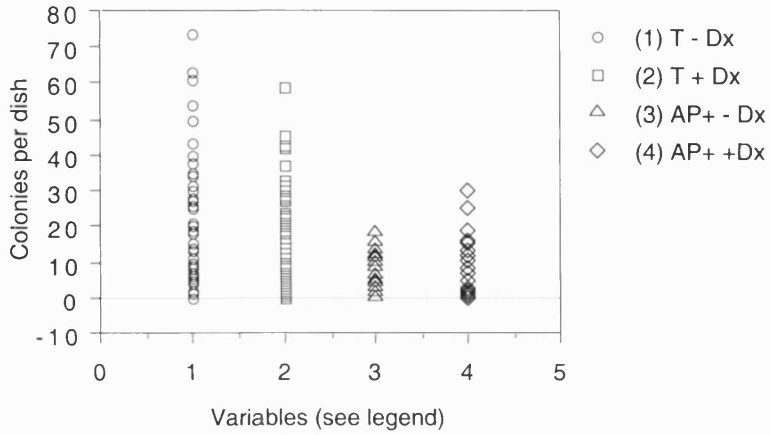
6.3.1 CFE

Analysis of the data of CFE for the whole population revealed a range in colony formation of 0-74 (mean = 21) and 0-59 (mean = 16) total colonies per dish, in the absence or presence of Dx, respectively. In the whole population Dx treatment decreased total colony formation by 24%, but increased the number of colonies expressing AP ~50% (figure 6.7). In the absence of Dx, the CFE of marrow derived from the whole population of female samples was significantly lower than that of the male samples ($p = .0317$, figure 6.8i). In contrast there was no difference in the number of AP colonies formed. When the growth characteristics of BMSC from female donors of $\geq$ 50 years of age were analysed independently, the CFE of the female samples was significantly lower than that of the male samples ($p = .0022$ and .0111, -/+ Dx respectively, figure 6.8ii). These comparisons demonstrate that the difference between the male and female populations was greater in the absence of Dx, than in its presence.

6.3.2 Proliferation

In the population as a whole (figure 6.9), treatment with Dx had no significant effect on the proliferation of BMSC in primary culture (mean change = 23%, $p = 0.276$). However, when the data were split by gender it was apparent that in the absence of Dx, proliferation of cells from female donors was significantly less than those from male donors (mean change = 47%, $p = .0347$, figure 6.10i). When the data for donors of age $\geq$ 50 years was analysed separately, the difference between males and females was significant in the absence or presence of Dx (mean changes of 69% and 73%; $p = .0134$ and .0274, respectively, figure 6.10ii).

Proliferation of BMSC from the whole population was reduced to 23% of total cell number with Dx treatment (figure 6.9). Furthermore, proliferation of female cells cultured in the absence of Dx was significantly less than that of male samples ($p = .0347$, figure 6.10i). When the growth characteristics of BMSC from female donors of $\geq$ 50 years of age were analysed independently (figure 6.10ii), the cell number of the female samples was significantly lower than that of the male samples. Again, this demonstrates the difference between the male and female populations was greater in the absence of Dx, than in its presence.

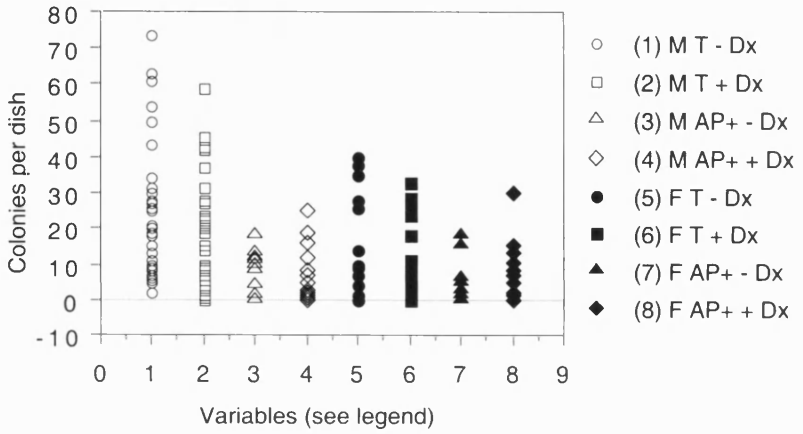


Group	(n)	Mean CFE +/-SE	CFE range	Mean rank
1	51	21 (+/- 2.5)	0-74	50.657
2	43	16 (+/- 2.3)	0-59	43.756
3	38	4 (+/- 1.0)	0-18	34.395
4	36	6 (+/- 1.3)	0-30	40.778

Figure 6.7 The effect of Dx treatment in a cohort study of total and AP colony formation

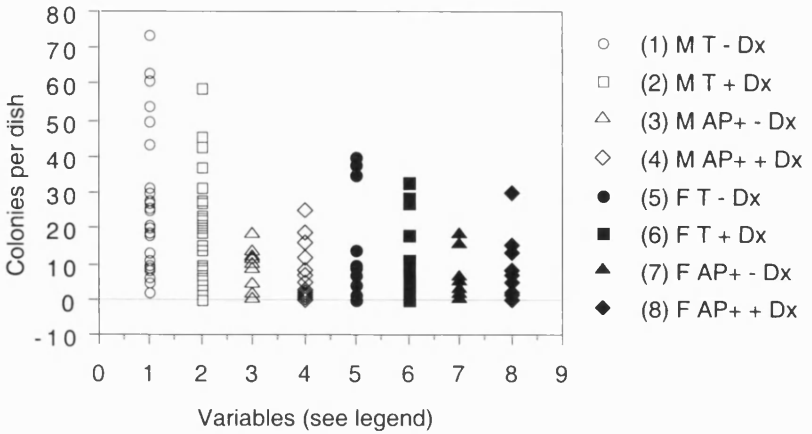
Results of total (T) and AP+ (AP+) colony formation were grouped into control and Dx treated cultures (-/+). The descriptive statistics for the data points in the univariate scattergraphs are shown in the table. These groups were compared using the Mann-Whitney U test. Results of the analyses are in appendix II.

(i) Whole population



Group	(n)	Mean CFE +/-SE	CFE range	mean ranks
1	34	25 (+/- 3.3)	2-74	33.765
2	28	20 (+/- 2.8)	0-59	28.750
3	22	5 (+/- 1.3)	0-18	20.341
4	21	7 (+/- 1.5)	0-25	23.738
5	17	11 (+/- 3.4)	0-40	17.176
6	15	9 (+/- 2.8)	0-33	15.733
7	16	3 (+/- 1.3)	0-18	14.438
8	15	5 (+/- 2.0)	0-30	17.667

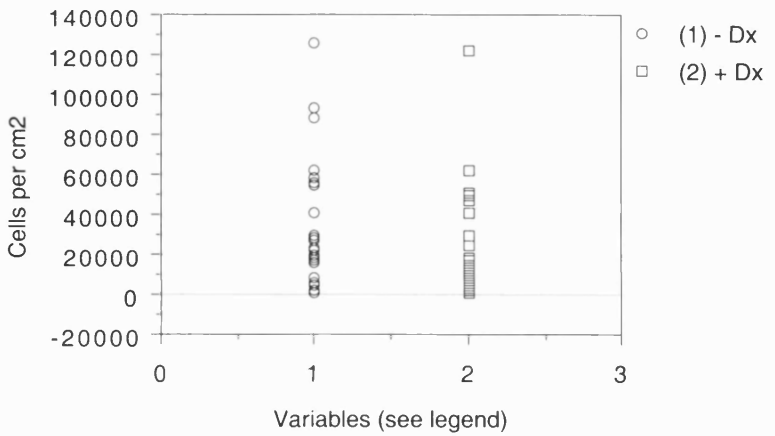
(ii) Population ≥ 50 years of age



Group	(n)	Mean CFE +/-SE	CFE range	mean ranks
1	30	24 (+/- 3.5)	2-74	29.083
2	24	19 (+/- 3.3)	0-59	25.521
3	20	5 (+/- 1.3)	0-18	18.475
4	19	6 (+/- 1.6)	0-25	21.605
5	17	14 (+/- 3.4)	0-40	17.412
6	15	12 (+/- 3.1)	0-33	16.562
7	16	4 (+/- 1.3)	0-18	15.912
8	15	6 (+/- 2.0)	0-30	18.156

Figure 6.8 The influence of gender and Dx treatment on total and AP colony formation

The results of colony formation (i, all donors, ii, samples from donors ≥50 years) were divided according to gender (M, male, F, female), and treatment with Dx (-/+). T, total and AP+, alkaline phosphatase positive colony formation.

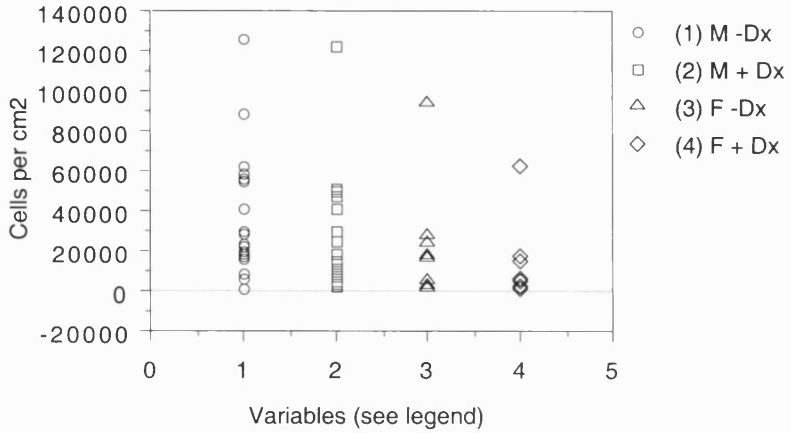


Group	(n)	Mean CN +/-SE	CN range	Mean rank
1	31	29772 (+/- 5431)	1390-126633	32.871
2	29	23153 (+/- 4900)	1230-123104	27.966

Figure 6.9 The effect of Dx treatment in a cohort study of BMSC proliferation

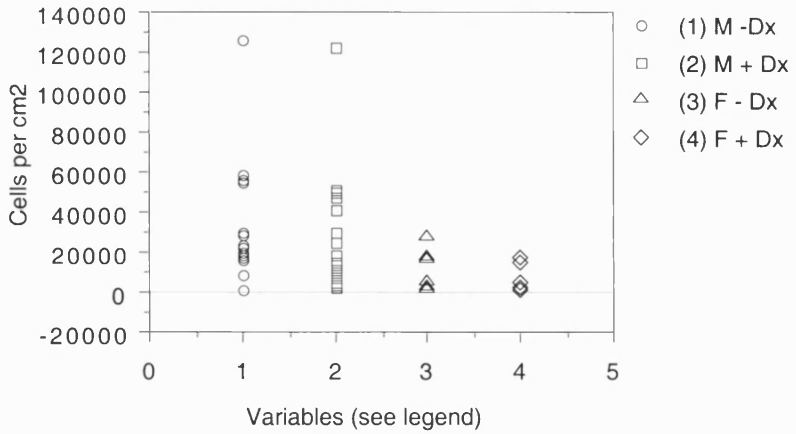
Results of BMSC proliferation were grouped into control and Dx treated cultures (-/+). The descriptive statistics are shown in the table. Results of the analyses are in appendix II. CN, cell number.

(i) Whole population



Group	(n)	Mean CN +/-SE	CN range	mean ranks
1	20	36210 (+/- 6996)	1764-126633	22.900
2	20	27287 (+/- 6390)	1280-123104	18.100
3	10	19045 (+/- 8804)	1390-93297	10.200
4	8	14103 (+/- 7345)	1230-63014	8.625

(ii) Population ≥ 50 years of age



Group	(n)	Mean CN +/-SE	CN range	mean ranks
1	16	32853 (+/- 7495)	1764-126633	19.000
2	17	27144 (+/- 7270)	1280-123104	15.118
3	7	10215 (+/- 3766)	1390-26937	7.857
4	6	7190 (+/- 3094)	1230-17969	6.000

Figure 6.10 The influence of gender and Dx treatment on BMSC proliferation

Results of BMSC proliferation (i, all donors, ii, samples from donors ≥50 years) were grouped according to gender, and treatment with Dx (-/+). M, male, F, female, CN, cell number.

6.4 Discussion

The influence of age on CFE and proliferation has been investigated in primary cultures of BMSC from a cohort of 56 donors (mean age of 61.8 years; range 15-84). Using this cell culture model we were unable to obtain evidence for the existence of an age-related decline in bone marrow CFU-F harvested from ribs, or in the proliferation of their progeny. This was true whether the population was treated as a whole or split by gender, and irrespective of the absence or presence of Dx. The demonstration of a lack of an association between donor age and the CFE or proliferation of the mesenchymal cells, may be of true biological significance and indicate that the number of CFU-F does not change with age in humans. These results are consistent with those published recently by Oreffo *et al* (1996, 1998) who also failed to detect an age-related decline in the CFE of human bone marrow harvested from the spine. They differ, however, from those of Majors *et al* (1997) who demonstrated a negative correlation between AP positive colony formation and donor age in human marrow harvested from the iliac crest.

These findings in human cell cultures are in marked contrast with those obtained using animal bone marrow in which it has been shown consistently that there exists an age-related decline in the number of CFU-F and/or nodule forming cells (Bab *et al*, 1984, Nimni *et al*, 1988, Tsuji *et al*, 1990, Quarto *et al*, 1995). In these experiments, the *in vitro* osteogenic maturation of the cells appeared to be normal, as AP continued to be expressed by the colonies, irrespective of donor age. This supports the proposal of Marie and De Vernejoul (1993) that differentiation of osteogenic precursors is normal in age-related osteoporosis.

The findings of this study do not preclude the possibility that a relationship between ageing and CFE (total or AP positive) does exist at other, more clinically relevant skeletal sites, for example the vertebra, or neck of femur (Davis *et al*, 1994, Bonjour *et al*, 1991). Our marrow samples were derived from rib, a non load-bearing site, which might not be as physiologically active as sites which have large strains imposed upon them. Site-related differences in human osteoblastic cell metabolism have been reported (Marie *et al*, 1992, Marie and De Vernejoul, 1993, Kasperk *et al*, 1995, Aerssens *et al*, 1997). Alternatively, our results may simply indicate that the changes in humans are less dramatic than those reported to occur in experimental animals. Also, in a study of human donors using surgical waste, many factors in addition to age may influence cellular activity including smoking, malnutrition, levels of exercise, endocrine status, drug history, underlying pathology, and race. These might influence the experimental outcome and confound interpretation of data.

A further possibility to explain these findings is that the decrease in bone formation observed *in vivo* may not occur as a consequence of a decrease in CFU-F number. A number of studies have found a reduction in the proliferation potential of human osteogenic cells with age (dePollak *et al*, 1997, Fedarko *et al*, 1992) and a decrease in the number of osteoblastic cells on the bone surface *in vivo* (dePollak *et al*, 1997, Shigeno and Ashton, 1995). The failure of the osteogenic population to expand appropriately may be due to impaired responsiveness to mitogens as suggested by Pfeilschifter *et al* (1993) and d'Avis *et al* (1997). Under standard culture conditions the response of CFU-F to growth factors is not apparent, but could be revealed by challenging BMSC with specific growth factors. A decrease in bone associated peptides, or reduced paracrine production of growth factors, could result in a remodelling imbalance, and contribute to the gradual loss of bone that occurs with age. Circulating levels of growth hormone and IGF-I have been observed to decrease with age (Bouillon *et al*, 1995) and to be decreased further in patients with idiopathic osteoporosis (Reed *et al*, 1995). Nicolas *et al* (1994) demonstrated an age-related decrease in the bone content of IGF-I, and in the ratio of stimulatory: inhibitory IGF binding proteins (IGFBP-5:IGFBP-3). Nicolas *et al* (1994) also reported an age-related decline in the TGFβ content in femoral cortical bone from both men and women.

Clearly insufficient samples were present in the perimenopausal population to make a critical analysis of this data alone. This study also emphasises that colony formation of individual donor samples is unpredictable, indicated by the many samples from which no colonies formed. In 28% of total donors samples cultured with Dx (3 samples of less than 30 years, and 9 of 57-84 years), less than 5 colonies formed per dish (i.e. 1 colony per 10,000 single cell seeded, or 1 colony per 4 cm^2 area). Likewise, the proliferation of these colonies was highly variable. As a large variation in inter-donor colony formation has been described (Majors *et al*, 1997, Muschler *et al*, 1997), a large number of donors are necessary in such a study to compensate for intrinsic errors. Also, from appendix III, it can be seen that the majority of patient samples used during the course of this research were suffering from lung cancer. A recent paper by Chasseing *et al* (1997) shows that this condition is correlated with a reduction in the colony forming potential of bone marrow. However, as the majority of patients were suffering from similar conditions the data are internally comparable, and the results valid.

Improvements to this study should include recruitment of larger number of normal donors, with marrow samples abstracted from a more clinically appropriate site such as the iliac crest. These volunteers could also be scanned to determine their bone mineral density, which could be correlated with the CFU-F census to determine the influence of age on either or both of these parameters. CFU-F census correlated with biochemical markers of bone turnover such as serum bone alkaline phosphatase (Duda *et al*, 1988) or osteocalcin

(bone formation, Price *et al*, 1980), urinary deoxypyridinoline crosslinks (Beardsworth *et al*, 1990) or tartrate-resistant acid phosphatase (bone resorption, Stepan *et al*, 1983) could indicate a relationship between the number of CFU-F observed *in vitro* and osteoblastic activity or the rate of bone turnover *in vivo*. In addition assessment of CFU-F by functional and immunochemical criteria such as STRO-1, with vascular cell adhesion molecule (VCAM-1, Benayahu *et al,* 1995), HOP-26 (Joyner *et al*, 1997) or MUC18 (Filshie *et al*, 1998) would allow the observed CFE to be compared with the expected CFE based on immunophenotyping.

An alternative hypothesis for consideration is that age-related osteopenia may not result from a decrease in absolute numbers of CFU-F, but from an imbalance in the proportion of progenitor cells that differentiate to form cells of other mesenchymal lineages as opposed to osteoblasts, for example adipocytes. This theory is supported by long standing observations of age-related changes in the composition of the marrow stroma including an increase in fatty tissue (Rozman *et al*, 1989), a concurrent decrease in osteoblast numbers (Uchiyama *et al*, 1994) and a marked increase in adipocytes in idiopathic osteoporosis (Meunier *et al*, 1971, Burkhard *et al*, 1987, Martin and Zissimos, 1991). The propensity of cells to enter the adipocytic lineage could be assessed by the proportion of colonies containing adipocytes following treatment of cultures with adipogenic agonists, for example thiazolidinedione (Ibrahimi *et al*, 1994), a ligand for the peroxisome proliferator-activated receptor γ (PPARγ) known to be of primary importance in the regulation of adipogenesis (Chawla *et al,* 1994, Tontonoz *et al,* 1994, Teboul *et al*, 1995, Nuttall *et al*, 1998).

Although no effect of ageing could be demonstrated in these studies further statistical analysis did reveal a significant effect of gender on CFE and on cell proliferation. Thus when compared with male samples, those from females were found to have significantly less total colony forming potential. Proliferation in cultures derived from female donors was also significantly decreased compared to that of male cultures. The difference in CFE and proliferation was independent of the absence or presence of Dx in donors ≥ 50 years. The number of AP positive colonies formed however, did not vary between male and female donors. This suggests that the proportion of colonies which express AP is greater in the female population than in the male. This is in contrast with recent report of Majors *et al* (1997) in which women older than 43 years were found to have a lower prevalence of AP positive colony forming units in marrow samples than males. Thus, marrow from female donors may contain more CFU-F which become committed to the osteogenic lineage, or CFU-F that begin to undergo osteogenic differentiation more rapidly.

It is probable that the reduction in total number of cells after 28 days culture is directly related to the reduction in colony formation, but whether this is a direct effect remains to be determined. Again, differentiation of a larger proportion of the cells in the female population may result in a lower proliferation rate, and fewer cells in the total population after 28 days in culture. In accordance with theories of Stein *et al* (1990), an upregulation of AP expression may be linked to a concurrent decline in preosteoblastic cell proliferation. This study may provide, therefore, tenuous evidence for premature differentiation and limited expansion of pre-osteoblastic cells in females, thereby reducing the number of cells capable of producing bone matrix. It also appears that the difference in the bone forming potential of the marrow is detectable at a very early stage in the cell life-span.

Chapter 7

Conditional immortalisation of BMSC with SV40 large T antigen

7.1 Introduction

Normal diploid cells in culture can only divide a limited number of times, during which they undergo many changes culminating in the cessation of cell division. This phenomenon of cellular ageing was first described by Hayflick (1965) and is now generally known as the Hayflick phenomenon. Cells undergo a process of ageing which is evident *in vitro* and have a limited lifespan of around 32 cumulative population doublings (Kassem *et al*, 1997). Immortalisation of mesenchymal cells overcomes this process of ageing, increasing the number of population doublings and delaying the onset of senescence. This significantly raises the number of cells available for analysis and allows investigation of the cell population over an increased length of time.

Transfection is an important laboratory tool which provides a means of introducing foreign DNA, normally in the form of a circular double stranded DNA vector, into proliferating cells *in vitro*. Immortalising vectors are integrating or episomally replicating DNA molecules that fulfil three specific requirements: they carry genetic material for expression of specific proteins, they contain origins of replication for both prokaryotic and eukaryotic replication, and they encode a drug resistance marker.

The prokaryotic selection marker DNA within the vector encodes resistance genes for antibiotic action. This allows large amounts of vector DNA to be generated following transformation of host bacteria such as *Escherichia coli* (*E. coli*) and manipulation and characterisation of the vector DNA to be undertaken rapidly. Another DNA sequence confers antibiotic resistance in the eukaryotic host and enables successfully transfected cells to survive in culture under conditions that are cytotoxic for the non-transfected population.

Immortalisation of transfected cells can occur through many molecular mechanisms. However, the plasmids chosen in this study, permutations of $pMT_1SV.neo$, act through expression of simian virus 40 large tumour antigen (SV40 large T Ag), an early protein originating from the monkey DNA tumour virus, simian virus 40. SV40 large T Ag is a 94 kilodalton oncoprotein, which alters host metabolic functions, primarily through binding to the tumour suppressors, retinoblastoma protein (Rb) and p53. These are recognised independently by different domains of the SV40 large T antigen (Stahl and Knippers, 1987). Wild type p53 and Rb are nuclear phosphoproteins involved in the control of cell-cycle progression. Sequestration of p53 prevents activation of target genes and results in DNA amplification. Similarly SV40 large T antigen binds and sequesters the non-phosphorylated form of Rb. This form of Rb is present in resting cells (G_0/G_1) where it inhibits activation of transcription and represses gene expression through binding to several proteins including E_2F, and C and D cyclins. Thus expression of SV40 large T Ag affects the cell-cycle control mechanism (Lewin, 1994).

The immortalising plasmid selected for initial evaluation in these studies was $pMT_4SV.neo$ (Peden *et al*, 1989) which has previously been used to immortalise human stromal cell lines (Cicuttini *et al,* 1992). The four synthetic repeats of the mouse metallothionein (MT) promotor present in this plasmid contain metal regulatory elements and are responsive to heavy metal cofactors including zinc (Zn^{2+}) and cadmium (Cd^{2+}). Supplementation of the culture medium with these cofactors (the permissive state) stimulates expression of SV40 large T Ag gene, removing normal constraints on the cell-cycle. In the absence of Zn^{2+} or Cd^{2+} the basal activity of the MT promoter, and thus SV40 large T Ag expression, is barely detectable (Peden *et al*, 1989), resulting in rates of proliferation and differentiation similar to those of non-transfected cells.

The most commonly used techniques for introduction of DNA into recipient mammalian cells are calcium phosphate-DNA co-precipitation, liposomal-mediated gene transfer and electroporation. However, it should be noted that irrespective of the method of introduction, the efficiency of stable transfection is determined by the cell type used, and may vary between different cell types by orders of magnitude. These methods are considered below:

Method	Description	Pros/Cons	Reference
Calcium Phosphate co-precipitation	Most widely used method. Endocytosis of co-precipitate into phagocytic vesicles.	Cheap. High efficiency. Does not work with all cell types.	Graham and van der Eb, 1973
Liposome mediated	Encapsulation of DNA within liposomes which fuse with the cell membrane.	Non-toxic. Does not interfere with normal physiological processes. Expensive.	Behr *et al,* 1989
Electroporation	Application of a brief high voltage electrical pulses to cells leads to formation of nm sized pored in plasma membrane through which DNA is taken up.	Causes damage to cells.	Smithies *et al*, 1985

The objectives of this work were to prepare large amounts of pure plasmid DNA suitable for transfection, and to optimise the transfection protocols.

Successful transfection was detected by monitoring cellular expression of SV40 large T Ag. A reporter plasmid, pCH110 (Pharmacia Biotech) containing the Lac-Z gene was co-transfected to facilitate optimisation of the transfection technique.

7.2 Results: Preliminary experiments

7.2.1 Preparation of DNA

The concentration and purity of plasmid DNA following purification using two commercially available kits are shown in table 7.1. The absorbance at 280 nm was equivalent to the protein concentration in the sample, a ratio of 1.8 represents a pure DNA sample.

Preparation	Plasmid	A_{260}/A_{280}	[DNA] (ng/µl)
Maxiprep Kit	$pMT_4SV.neo$	2.16	378
	$pMT_4SV.neo$	1.87	843
	pCH110	1.77	1410
Qiagen kit	$pMT_4SV.neo$	1.8	1279
	pCH110	1.8	422.5

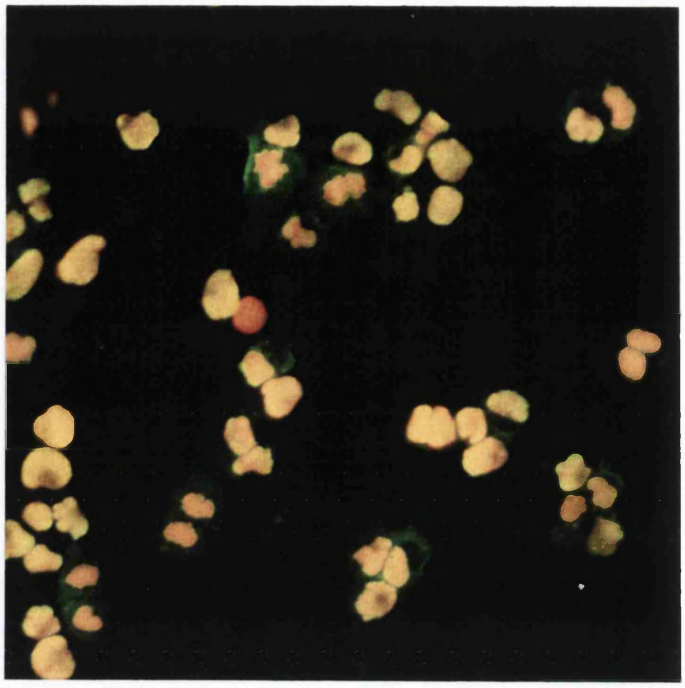
Table 7.1 Results of purification of plasmid DNA from E. coli.

A substantial amount of DNA was purified. The purity of DNA extracted using a Qiagen kit was better than using a Maxiprep Kit.

7.2.2 Detection of SV40 large T Ag in a positive cell-line

As a preliminary investigation it was necessary to determine whether cellular expression of SV40 large T Ag could be detected by immunocytochemistry using the monoclonal antibody pAb101. $MRC_5SV_1TG_1$ cell cytospins (figure 7.2) stained positive for SV40 large T Ag. Using the same antibody expression of SV40 large T Ag was also detected in cell lysates of $MRC_5SV_1TG_1$ immobilised on nitrocellulose (figure 7.3). The antigen was detected in amounts of total protein above 100 ng, whereas only non-specific Ab binding occurred in the MG-63 samples.

i)



ii)

Figure 7.2 Expression of SV40 large T antigen by $MRC_5SV_1TG_1$ cells

$MRC_5SV_1TG_1$ and CHO cells were harvested and fixed on glass slides (section 2.8.1). A FITC conjugated anti-mouse IgG was used to immunolocalise bound 1° antibody. Cells were counter stained with 0.1% ethidium bromide solution. i) Green fluorescence in $MRC_5SV_1TG_1$ cells highlights expression of SV40 large T antigen. ii) CHO cells were used as a negative control.

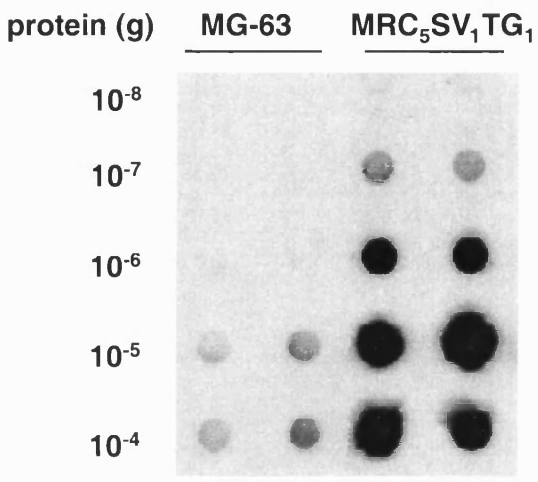


Figure 7.3 Detection of SV40 large T Ag in positive and negative cell lysates

MG-63 cells and $MRC_5SV_1TG_1$ cells were lysed and soluble protein dot blotted onto a cellulose acetate membrane. Membranes were probed with pAb101 mAb (5 μg/ml in PBS). Bound antigen was detected using enhanced chemiluminesence, and exposure to a photosensitive film for 30 seconds.

7.2.3 Electroporation of cells with $pMT_4SV.neo$

Initial attempts to introduce plasmid DNA into BMSC by methods of calcium phosphate co-precipitation and liposome-mediated transfection were unsuccessful. The potential of transfection by electroporation was therefore explored. Electroporation does not have the limitations of chemical treatment, and can be used for stable transfection of cell types which may normally be refractory to other methods. However, in preliminary investigations the cells subjected to electroporation did not proliferate when replated onto plastic, indicating that cell viability was substantially reduced using this method. It was known that for many cell types optimal transfection occurs at an electroporation field strength that results in cell death of 50% or more, so it was of importance that the conditions of electroporation were optimised (Andreason and Evans, 1988).

7.2.4 Optimisation of electroporation conditions

The critical factor in electroporation is the time constant. This can be altered by varying the four key parameters; the field strength (which is a function of the capacitance, distance between electrodes, and the voltage applied), the duration of the current pulse, the pulse shape, the conductivity of the electroporation buffer (type and volume), the DNA concentration and the cell concentration. It is possible to increase the time constant by increasing the voltage or capacitance across the cuvette or by reducing the volume of the cell suspension as these are directly and inversely related respectively. The equation describing these properties is shown below:

$$\text{Time Constant (ms)} = \frac{\text{Potential difference (V)}}{\text{Current (I)}} \times \text{Capacitance (F)}$$

Experiments were carried out varying the voltage, capacitance and suspension volume as above in an attempt to achieve different time constants. Experimental conditions are shown in table 7.4 and results in table 7.5.

Conditions	Experiment			
	1	2	3	4
Cell type	CHO	MG-63	CHO	MG-63
Buffer	PBS	PBS	HBS	HBS
Volume of Cell Suspension (μl)	200	500	500	500
Fixed Capacitance (μF)	125	125	250	125
Potential Difference (V)	300	200, 300	300, 350	300, 350
Plasmid (10μg/ml total)	$pMT_4SV.neo$	$pMT_4SV.neo$ pCH110	$pMT_4SV.neo$ pCH110	$pMT_4SV.neo$ pCH110

Table 7.4 Initial experimental conditions of electroporation

Voltage	Time Constants CHO	MG-63
200 V		3
300 V	3.2, 3.7	3.3, 4.8
350 V	5.8	5.3

Table 7.5 Summary of time constants resulting from the above experiments

To gauge the extent of cell poration and viability, an aliquot of electroporated cells was stained with propidium iodide (PI) and fluorescein diacetate (FDA). PI is only taken up by cells of which the cell membrane has been rendered permeable, FDA is taken up into viable cells by endocytosis. Uptake of the fluorescent markers was analysed by FACS analysis. CHO and MG-63 cells were resuspended at $1x10^7$ cells/ml in HBS and electroporated as in section 2.11.6, with modification to the variables shown in table 7.6.

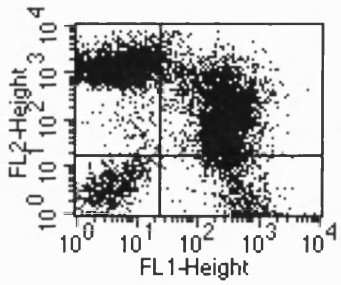
Variables	Constants
volume in cuvette	HBS buffer
voltage	10^7 cells /ml
capacitance	electrode distance, 0.4 cm

Table 7.6 Properties of electroporation

Examples of FACS analysis of viability and poration following electroporation of MG-63 and CHO cells are shown in figure 7.7. Importantly, viable, porated cells are represented in the upper right quartile of the scatter plot. The FACS plots shown in figure 7.7 represent the same experiment as the bars indicated with an asterisk in figure 7.8, which shows the results of the percent poration of viable cells gained from FACS analysis overlaid with the results of the respective time constant attained.

Figure 7.7i demonstrates that although 86% of the CHO cells were porated, only 40% of the porated cells remained viable. Of the MG-63 cells, 98% were porated, but again only 40% of the total population remained viable following electroporation. Thus, a viability of ~50% of the cells was maintained.

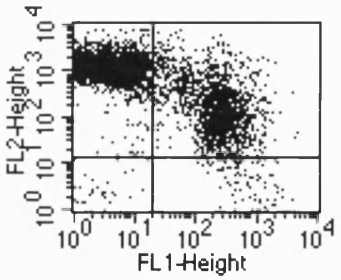
i)



300 µl, 125 µF, 300 V

Quad	Events	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	4534	45.34	6.63	5.30	1302.14	1111.32
UR	4091	40.91	310.39	236.82	374.56	156.70
LL	658	6.58	4.92	3.99	4.71	3.79
LR	717	7.17	577.24	419.65	7.03	4.57

ii)



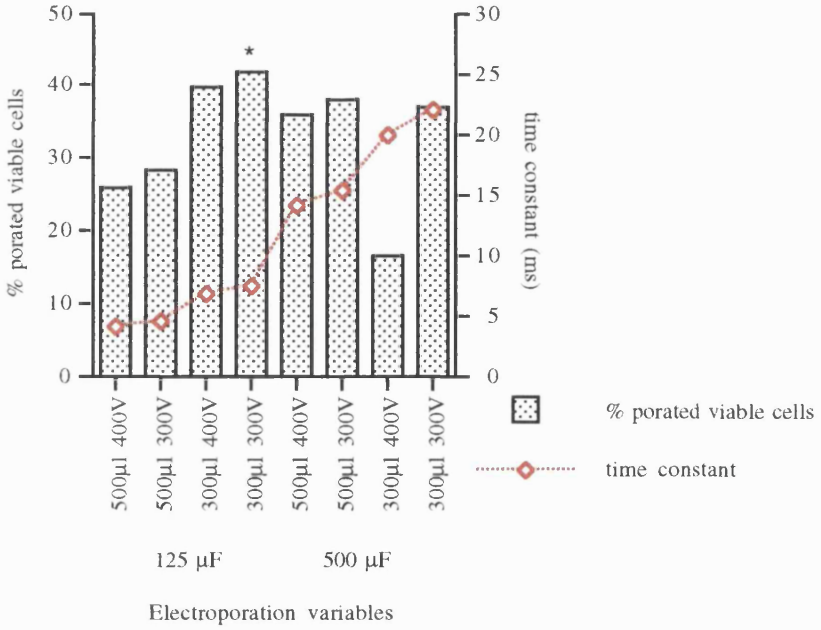
300 µl, 125 µF, 400 V

Quad	Events	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	2903	58.06	4.88	3.78	1370.34	1172.16
UR	1964	39.28	286.07	198.37	377.79	162.46
LL	30	0.60	4.17	2.92	4.06	3.08
LR	103	2.06	819.65	594.98	6.70	4.97

Figure 7.7 Viability and poration of CHO and MG-63 cells following electroporation

CHO i) and MG-63 ii) cells were electroporated under the conditions of volume, capacitance and voltage shown. Cells were stained using the method in section 2.11.6. UL (upper left) quartile indicate porated, but dead cells, UR (upper right) quartile: porated, but live cells, LL (lower left):, non-viable unporated and LR (lower right): viable, unporated cells.

i)



ii)

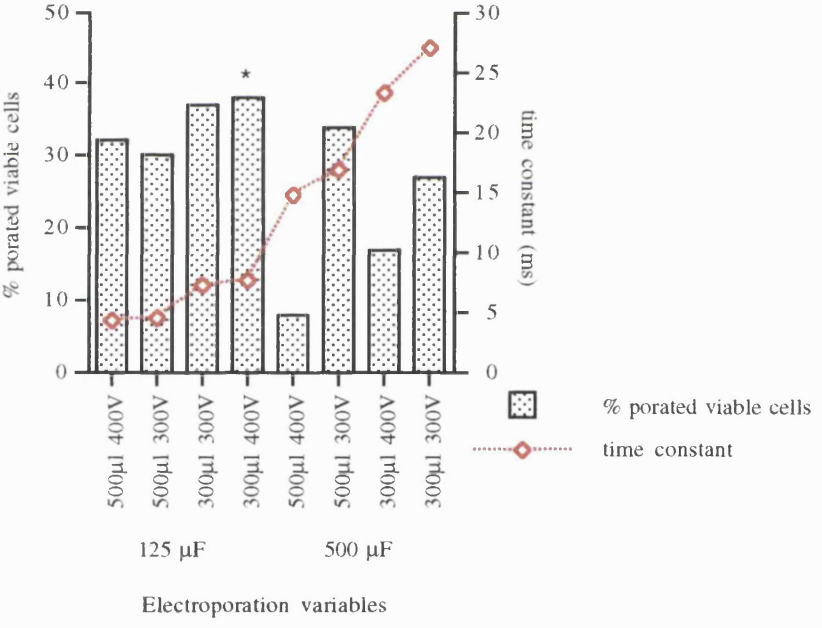


Figure 7.8 Poration and cell viability following electroporation

i) CHO and ii) MG-63 cells were electroporated under different conditions, and the time constant recorded. An aliquot of cells was analysed for poration and viability by FACS analysis. The histograms show the percentage of porated, viable cells derived from FACS results, plotted in the order of increasing time-constant (overlaid line-graph). The FACS data corresponding to the columns marked with an asterisk are shown in figure 7.7.

Figure 7.8 shows that the most suitable conditions for electroporation of CHO or MG-63 cells were 125 μF, 300 μl and 300 or 400 volts respectively. These conditions resulted in high levels of viability and poration, with a time constant of 7 ms for both CHO and MG-63 cells. Of the variables tested, the capacitance had the greatest effect on the time constant. 125 μF consistently produced the greatest percentage of porated cells whilst maintaining viability. A volume of 300 μl was found to be preferable to 500 μl, and the difference between 300 and 400 volts was negligible.

Following optimisation of the electroporation method with these cells, immunocytochemistry of SV40 large T Ag in MG-63 and CHO cell cytospins showed no fluorescence, and thus transfection appeared to have been unsuccessful. Even when the transfected CHO cells were treated with various concentrations of Zn^{2+} for 7 days, SV40 large T Ag was not expressed (figure 7.9). Similarly, cells did not survive in selection medium or express β-galactosidase when cells were transfected with pCH110 using these conditions. Further electroporations were carried out using the conditions established in these experiments, but without success.

7.2.5 Transfection of BMSC

Using the conditions evaluated for MG-63 and CHO cells, even though transfection had not been successful, an attempt was made to transfect BMSC. When transfected with reporter plasmid, BMSC were found to express β-galactosidase as shown in figure 7.10. However, negative control cells also stained blue. Thus, it could not be concluded that the peptide expressed by the cells was translated from the plasmid DNA.

Following transfection with $pMT_4SV.neo$, BMSC cultures maintained for one month in the presence of selection medium and Zn^{2+}, contained a mixture of cell debris and small spherical structures that contained densely packed cells, presumably of clonal origin (figure 7.11). These structures were harvested, and disaggregated by repeated passage through a 0.8 mm needle (Becton Dickinson) and then replated into 96 well microtitre plates, but none of the cells isolated in this way gave rise to cultures.

7.2.6 Characterisation of transfected human bone-derived cells

Despite these initial difficulties, several strains of SV40 transfected cell lines were eventually obtained (unpublished data; K. Stewart, N. Fairweather and C. Jefferiss). Initial characterisation studies of the cells are described below.

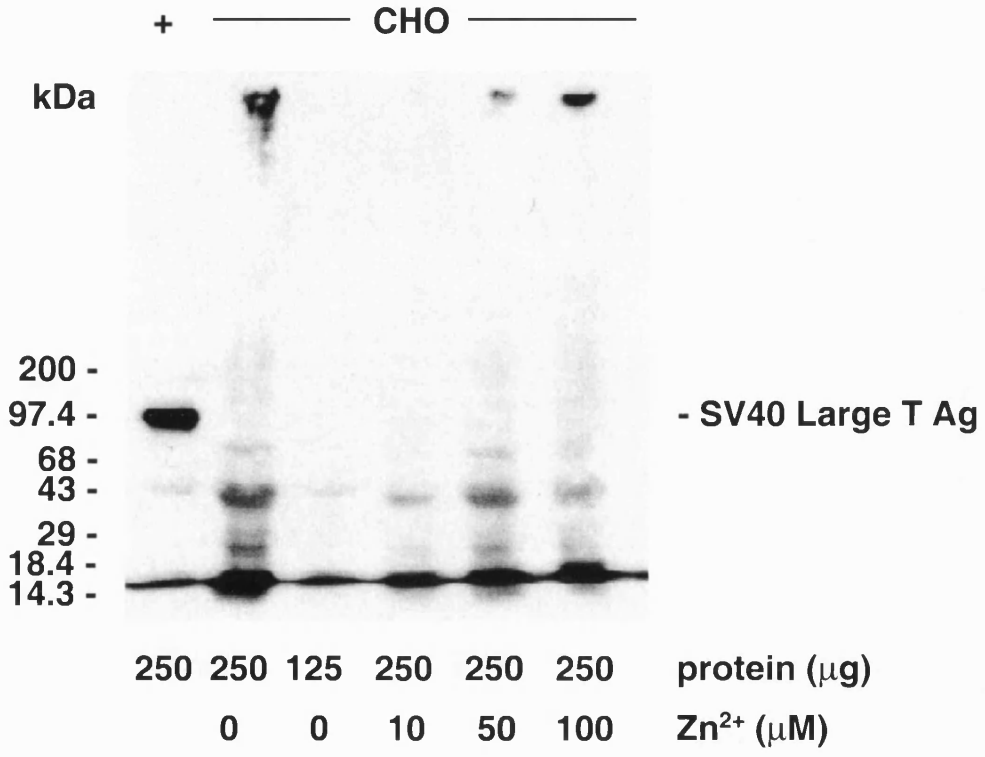


Figure 7.9 Lack of expression of SV40 large T Ag in transfected CHO cells

Following electroporation CHO cells were cultured for 72 hours -/+ Zn^{2+} at various concentrations, then harvested and lysed. Cell lysate was loaded onto a 7.5% PAGE gel in the amounts shown, and blotted onto cellulose acetate. Fixed protein was probed with pAb101 (5μg/ml) antibody, and bound antigen was detected using enhanced chemiluminesence, and exposure to a photosensitive film for 30 seconds. SV40 large T Ag was detectable in the positive control lane (+, $MRC_5SV_1TG_1$ protein lysate) only.

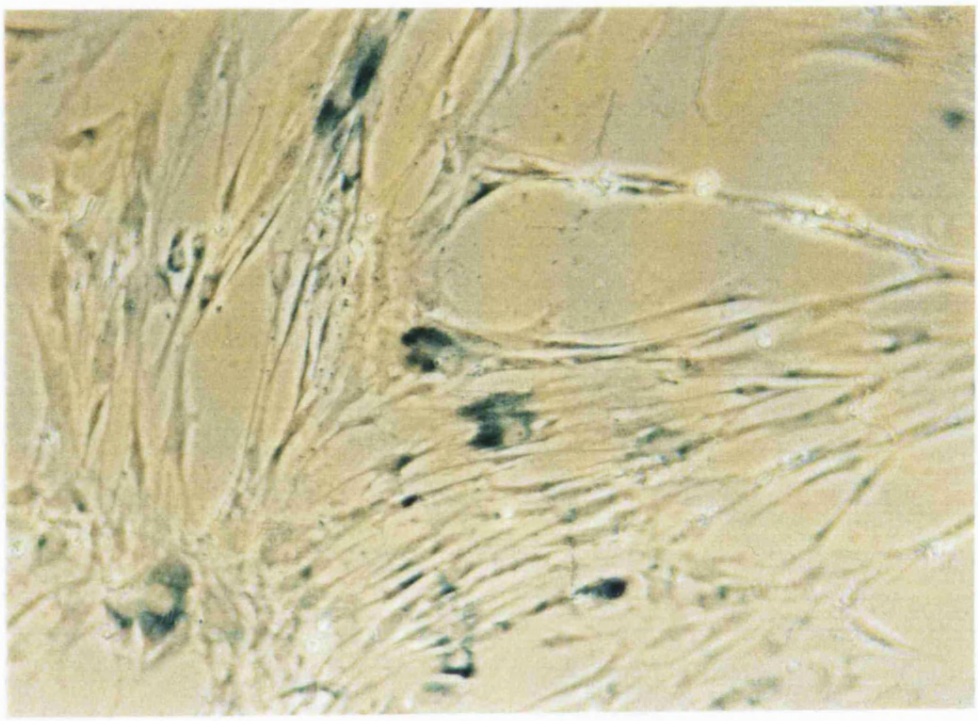


Figure 7.10 ***Positive β-galactosidase stain in BMSC cells transfected with pCH110***

BMSC were electroporated with pCH110 and then cultured for 72 hours. Cells were fixed and stained with X-Gal following the protocol in section 2.11.7. Cells which stained blue were positive for β-galactosidase expression. Cells were observed under a light microscope (x 100).

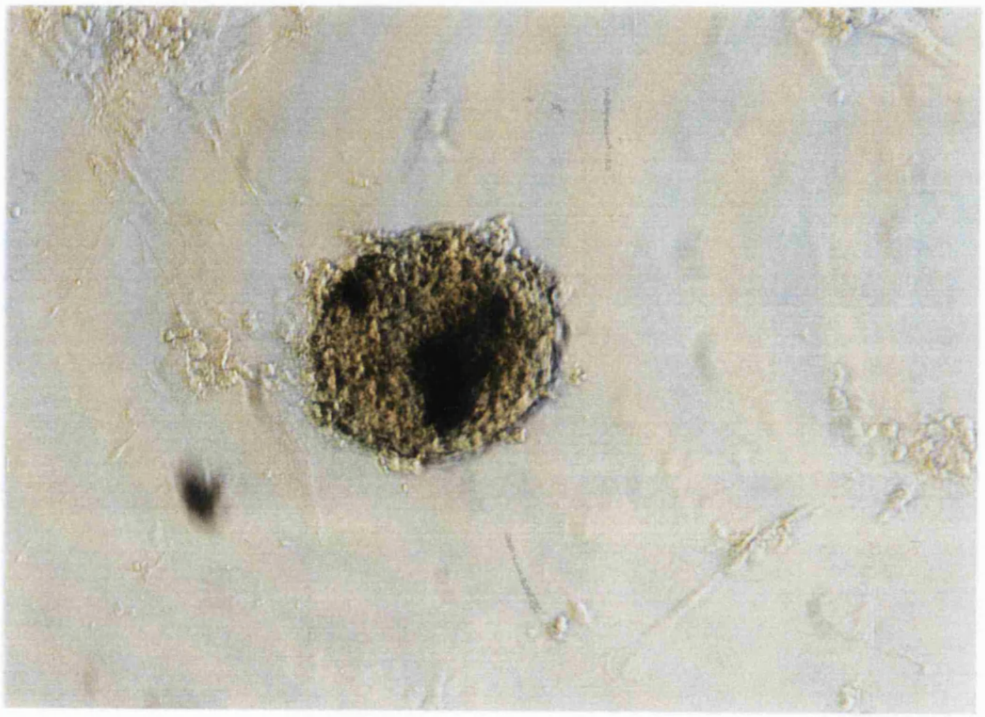


Figure 7.11 Ball of cells formed in transfected cell cultures post-electroporation

BMSC were electroporated with $pMT_4SV.neo$ and then reseeded in culture. Cells were maintained for one month in selection medium containing G418-S, Zn^{2+} and Cd^{2+}. During this time balls of cells developed (observed at x 100 magnification). These were surrounded by cell debris.

7.2.7 Cell morphology

A striking feature of these cell strains was the change in morphology and growth kinetics when compared to the parental cells. Initially the cells grew into clumps, or spheres, from which spindle-shaped cells migrated out from the dense mass (if this remained attached to the substratum). The cells then proliferated to form a monolayer, which became multilayered over 14 days. With more time in culture, the cells lost contact inhibition and continued to proliferate post-confluence figure 7.12.

7.2.8 Expression of SV40 large T Ag

An initial experimental requirement was to determine whether SV40 large T Ag was synthesised by the transformed cells, and to eliminate suggestions that spontaneous immortalisation had occurred, or that the cells had been contaminated with cells of a cell-line. A dot blot (figure 7.13) demonstrated that SV40 large T Ag expression was detected in 50 μg total protein of cells (patient #546 transfected with $pMT_1SV.neo$). SV40 large T Ag was also detected in 10 μg of $MRC_5SV_1TG_1$ cells, but was not detected in SaOS-2 cell lysates. Thus the cells were definitively transfected with immortalising plasmid.

7.2.9 Control of SV40 expression by heavy metal control

Regulation of SV40 large T Ag expression by induction by the pMT promotor was established by culturing the transfected cells in the absence or presence of 200 μM $ZnCl_2$ and/or 1 μM $CdCl_2$. When compared with extracts prepared from the control cell-line (SaOS-2), the expression of SV40 large T Ag was barely detectable in cells cultured in control medium (figure 7.14). The addition of Zn^{2+} or Cd^{2+} resulted in a significant increase in SV40 large T Ag expression, which was increased further when both agents were added in combination. The amount of protein synthesised was greater than that of cells transfected with pSV_3.neo in which expression of SV40 large T Ag is controlled by a wild type promotor and is therefore constitutive.

Treatment	$pMT_1SV.neo$				pSV.neo	+	-
	Mg^{2+}	Zn^{2+}	Cd^{2+}	Zn/Cd			
Relative OD (T/C)	1	18	26	46	27	67	0

Table 7.15 SV40 large T Ag expression after treatment with heavy metals

Relative optical density (OD) of bands on western blot in figure 7.10, compared when OD of control (200 μM $MgCl_2$) treated cells = 1.

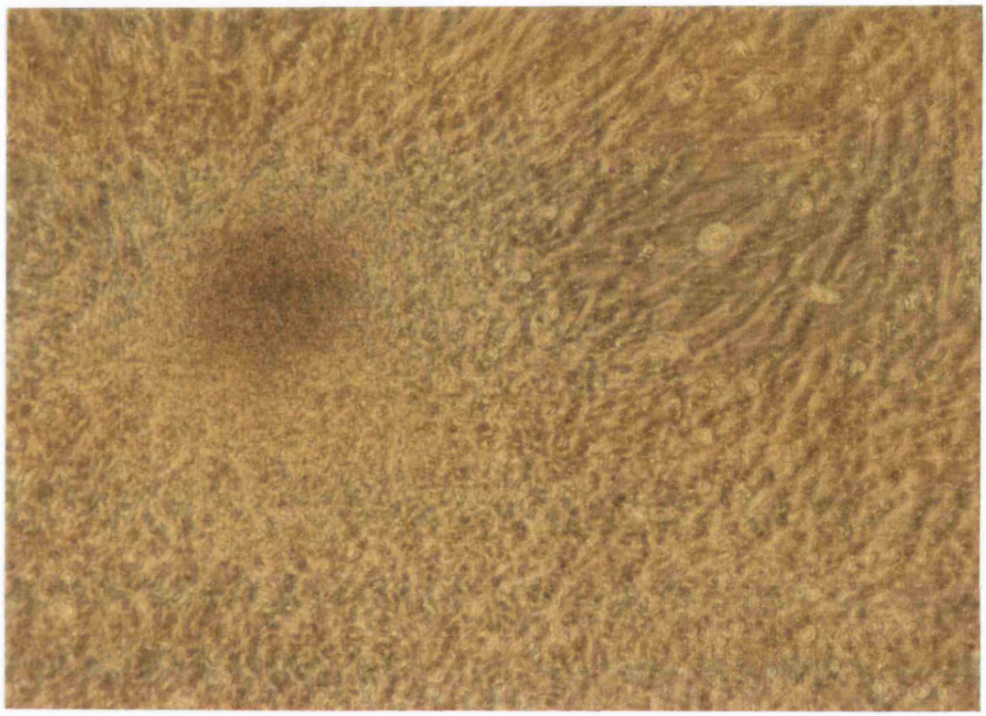


Figure 7.12 Growth of cells transformed with SV40 large T antigen.

Trabecular cells from patient #546 were transfected with $pMT_1SV.neo$. Cell cultures were maintained in medium supplemented with G418-S, Zn^{2+} and Cd^{2+}. After 6 weeks cells began to proliferate and formed cell monolayers with areas of cells at high density. In a few cultures cells became multilayered and formed nodule-like structures (as seen in the photograph). The culture was observed under a light microscope at x 100 magnification.

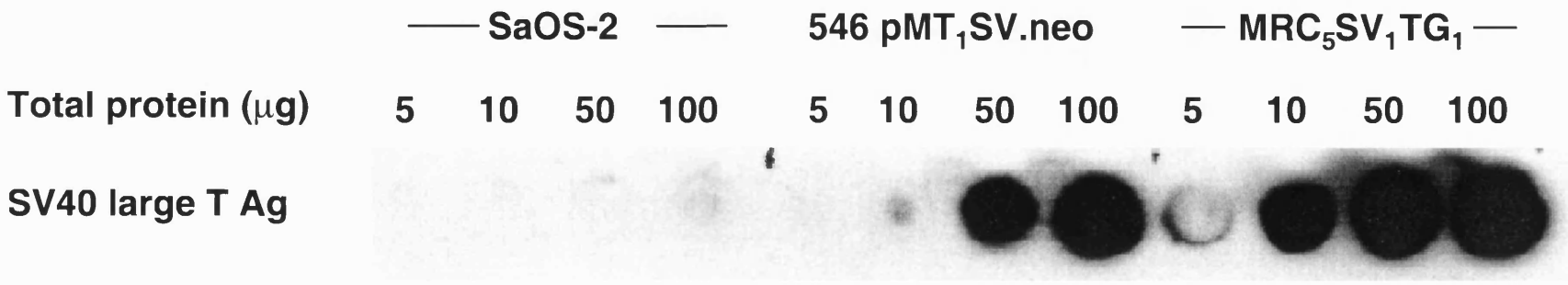


Figure 7.13 Detection of SV40 large T Ag in human HBDC transfected with $pMT_1SV.neo$

Various amounts of protein of #546 cells transfected with $pMT_1SV.neo$ (transfected cells), SaOS-2 (negative control) and $MRC_5SV_1TG_1$ (positive control) whole cell lysates were applied onto cellulose acetate membrane. Antigen was immunolabelled with anti-SV40 large T Ag and bound antibody was detected by enhanced chemiluminesence and exposure to a photoreactive film for 30 seconds. SV40 large T Ag was detectable in 50 μg of the transfected cells lysate, and 10 μg of the positive cell line.

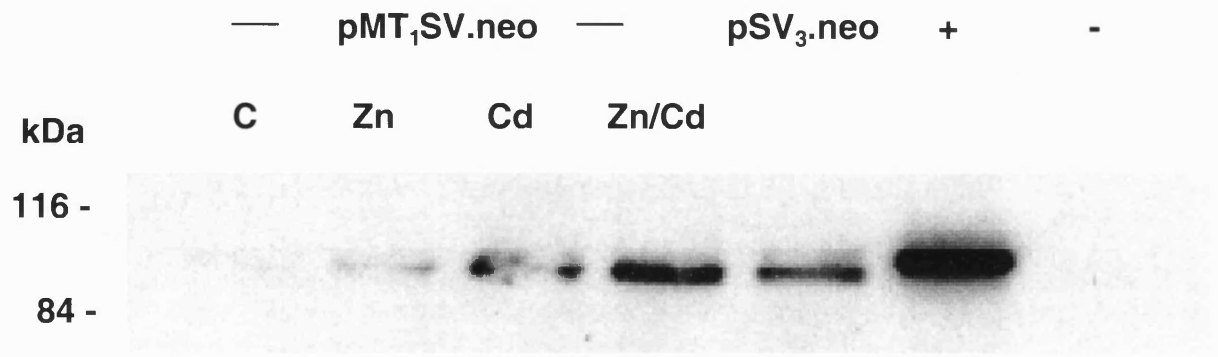


Figure 7.14 Detection of SV40 large T Ag in transfected human HBDC

#546 cells transfected with $pMT_1SV.neo$ were cultured in the presence and absence of 200 μM $ZnCl_2$ and/or 1 μM $CdCl_2$ for 7 days. Control cells were treated with magnesium chloride (200 μM). These cells and $pSV_3.neo$ transfected cells were harvested and lysates prepared. 50 μg protein was separated on a PAGE gel and fixed onto cellulose acetate membrane by western blotting. Antigen was immunolabelled with pAb101, and bound antibody was detected by enhanced chemiluminesence. SV40 large T Ag was detected in conditionally immortalised cells treated with heavy metals and cells immortalised with plasmid containing the wild type promotor, and the positive control cell line ($MRC_5SV_1TG_1$). The antigen was not detected in the negative cell line (SaOS-2).

The intensities of the SV40 bands demonstrate numerically an increase in antigen expression through Zn^{2+} and Cd^{2+} treatment and a co-operative response when cells were treated simultaneously with the two factors.

7.2.10 Alkaline phosphatase expression and mineralisation

The ability of the transfected cells to express AP and deposit mineral was investigated in cultures treated with or without Dx. AP was expressed in all cultures which had been treated with Dx (an indicative culture is shown in figure 7.16) irrespective of the presence of heavy metal cofactors. In these cultures, Fast-red TR stain was localised to areas of high cell density. These areas also contained mineral deposits as demonstrated by von Kossa staining.

7.2.11 Characterisation of osteoblastic lineage markers AP and STRO-1

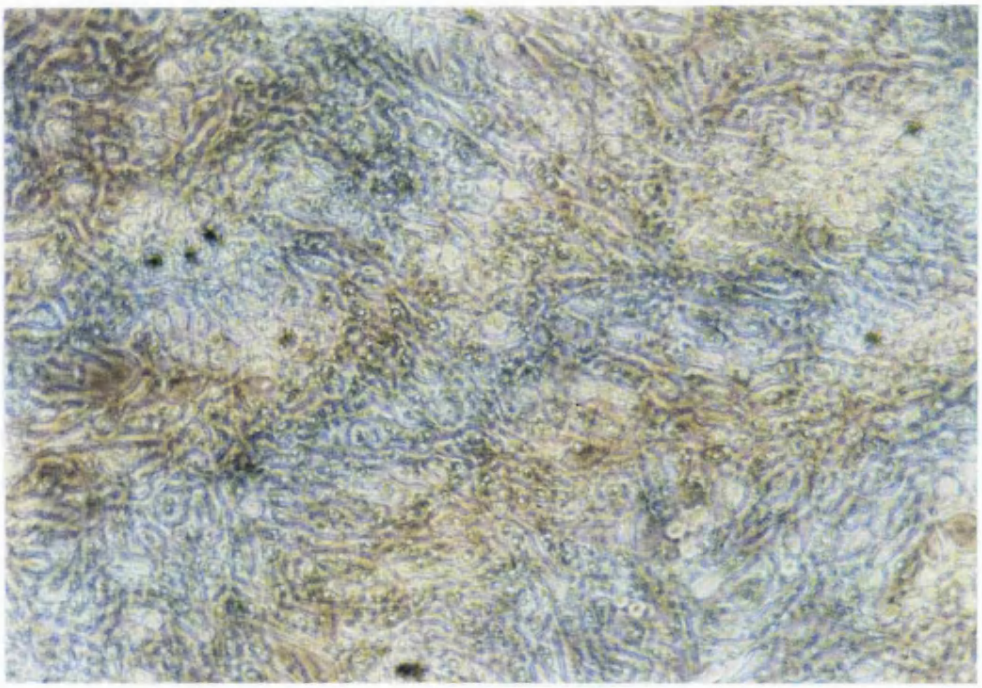
Cell surface expression of STRO-1 and AP was inspected by dual fluorescent staining and FACS analysis on cells cultured with or without Dx. The FACS histograms representative of the results of #546 $pMT_1SV.neo$ (passage 6) shown in figure 7.18 are also summarised in table 7.17.

Transfectant	Passage #	Surface expression STRO-1	Surface expression AP	Effect of Dx STRO-1	Effect of Dx AP
546pMT$_1$SV.neo	6	*	******	+	++
546pMT$_1$SV.neo	11	*	*	+	+

Table 7.17 Summary of STRO-1 and AP expression of immortalised human bone cells.

Analysis by FACS revealed that the $pMT_1SV.neo$ transfected cells (#546) expressed STRO-1 and AP on their cell surfaces, and these were recognisable by the monoclonal antibodies post-transfection. Only low levels of STRO-1 were detectable. At passage 6, $pMT_1SV.neo$ expressed AP in abundance. However, AP expression decreased with an increase in passage number. Treatment with Dx increased surface expression of STRO-1 and AP, although this had a greater affect on AP expression at passage 6 than 11.

i)



ii)

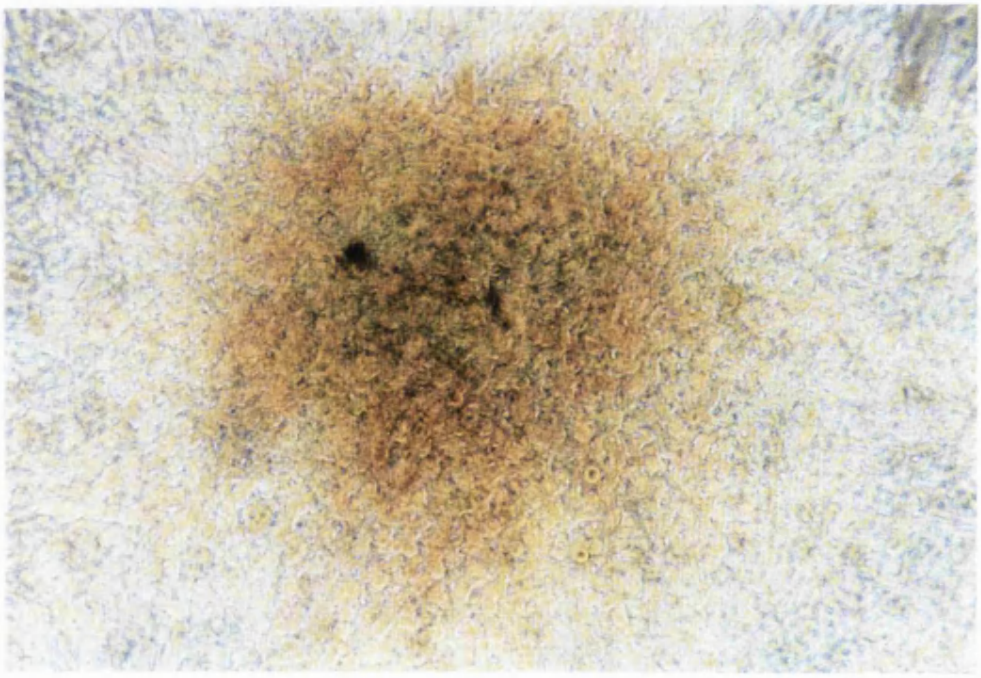
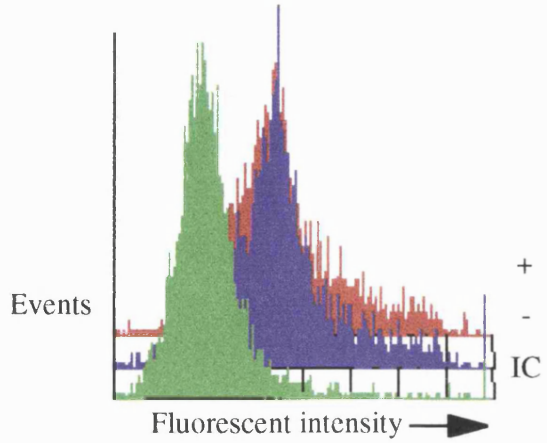


Figure 7.16 AP expression and mineral deposition in cells transfected with $pMT_1SV.neo$

#564 cells transfected with $pMT_1SV.neo$ were cultured in medium containing Zn^{2+} and Cd^{2+} and treated without (i) or with (ii) Dx for 5 days. Cultures were then fixed with NBF and stained with Fast-red TR and von Kossa stain. Cells were observed at x 40 magnification.

i)



ii)

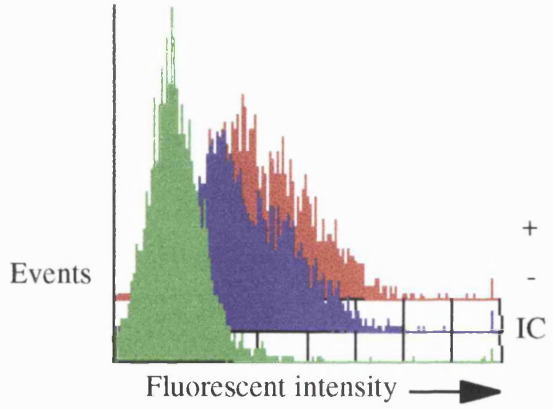


Figure 7.18 Expression of STRO-1 and AP on #546 pMT$_1$SV.neo cells

#546 pMT$_1$SV.neo cells (passage 6) were treated in the absence or presence (-/+) and without Dx (-) for 5 days. Cells were dual stained with STRO-1 (i) and B4-78 (ii), and labelled with RPE and FITC conjugated secondary antibodies. An isotype control antibody was used as a negative control (IC). The cells expressed both STRO-1 and AP antigens on their surfaces.

7.3 Discussion

Investigation of bone marrow stromal cells in culture is limited by their inherent tendency to differentiate and/or become senescent after a limited number of population doublings. It was therefore considered advantageous to immortalise primary BMSC and generate cell lines. After much trial and error, adherent cells derived from human bone marrow stroma were successfully transformed with SV40 large T Ag present in a conditionally regulated plasmid. These were selected in medium containing G418-S and have now undergone twelve passages post-transformation. To date nine lines have been transformed.

Initial attempts showed the marrow-derived cells to be resistant to transfection using calcium phosphate and Lipofectamine. In retrospect, the difficulties experienced early on were probably due to the age of the patients from whom the marrow samples were taken. The samples used were derived from a female donor of 57 years, and two males of 63 and 74 years. BMSC from these donors grew slowly (visual observation), and were in culture for 6-8 weeks compared to the usual 4-6 in order to generate sufficient cells for transfection. In addition these donors were diagnosed as having lung cancer, and it has been shown recently (Chasseing *et al*, 1997) that this is associated with a significant reduction of CFE and proliferation of BMSC *in vitro*. The success of these methodologies is dependent on the cells actively dividing to ensure expression of the SV40 large T Ag. Consistent with the age and underlying pathology of the original donor being confounding factors, the cultures that were successfully transformed were derived from younger male patients (#546 was 15 years old, and #532 was aged 56) who were not suffering from any form of malignancy.

Another factor which may have hampered the transfection efficiency may have been an abundance of extracellular matrix in these cultures. The highly negative charge of DNA may have resulted in binding to polypeptide moieties in the extracellular matrix, reducing its availability for transferral into cells. To limit this effect, adherent cultures were treated with collagenase prior to transfection, and cell suspensions were washed thoroughly before electroporation. The absence of SV40 large T Ag expression after transfection of MG-63 and CHO cells, however, remains baffling. Either the plasmid DNA did not enter the cells, or SV40 large T Ag was not expressed by the cells. It was recently observed however, that MG-63 cells behave in an unpredictable manner following transfection, failing to express SV40 large T Ag when transfected with immortalising plasmids alone or co-transfected with a reporter construct (J. Hodgekinson and P. Sharpe, unpublished data; A. Grigoriadis, personal communication). Expression of β-galactosidase by negative control mesenchymal cells (nontransfected cells) is suggested to be due to a high incidence of senescent cells in these cultures which were derived from elderly donors. Senescent human mesenchymal cells are known to express a novel form of β-galactosidase (Dimri *et al*, 1995).

Characteristic changes were observed in cells transformed with SV40 large T Ag. On observation by microscopy, the cells had altered growth characteristics, their morphology changed from spindle-shaped to an epithelial-like cell morphology, and they were noticeably reduced in size. Contact inhibition of the cells was lost, and when confluence was reached the culture became multilayered. These are all reported characteristics of immortalised cells cultured *in vitro* (Darnell *et al,* 1986). Additionally, the proliferation rate of the cells was much increased, the cells required passaging at three day intervals, rather than the usual 1-2 weeks required in non-transformed BMSC cultures.

On primary analysis, these cells had an phenotype similar to osteoprogenitor cells with an ability to express AP and STRO-1, and deposit a mineralised extracellular matrix in response to Dx treatment. AP expression also appeared to be induced by increased cell density. Expression of SV40 large T antigen was controllable by the addition of Zn^{2+} and/or Cd^{2+} to the culture medium, and the proliferation rate was noticeably reduced in cultures maintained in the absence of cofactors. Future investigation requires identification of the characteristics of the cells cultured in the absence or presence of cofactors.

These cell lines are presently strains. Due to the nature of the experimental protocol and the inherent multipotential properties of CFU-F, cells from a number of heterogeneous colonies were pooled and transformed as a single population. Thus the lineage, age and differentiation state of the cell, or indeed the number of different cells, that was/were transformed is unknown. In essence, these cultures are expected to consist of a heterogeneous population. It is thought that the cultures will metamorphose with an increase in passage number. With time in culture, cells of a particular phenotype and possibly a greater proliferative potential, may become dominant. Also, the dominant phenotype may change with passage number due to differentiation, de-differentiation, or as a result of other culture conditions such as cell density or presence of growth factors. Once the *in vivo* potential of the cells to form mineralised tissue has been determined, and the multipotentiality of each of the lines investigated, the cells will be cloned in the hope of obtaining a mesenchymal cell lines that differ in their potential for proliferation and differentiation.

Conditional immortalisation has been successfully used to develop several osteoblastic cell lines. These include lines from BMSC primary cultures derived from the iliac crest (Hicock *et al,* 1998) and rib (Houghton *et al,* 1998), and from osteoblasts derived from human foetal trabecular bone (Harris *et al,* 1995) and from elderly donors (Bodine *et al,* 1996). Two of these lines are reported to be bipotential with the ability to differentiate into osteoblasts or adipocytes under specific culture conditions (Hicock *et al,* 1998, Houghton *et al,* 1998). However, all of these lines were immortalised with a gene coding for a temperature-sensitive (ts) mutant of SV40 large T antigen which is expressed at a permissive temperature of 34°C.

The return to a nonimmortalised state can be induced by incubating the cells at a restrictive temperature of 39.5°C. Temperature sensitive immortalisation thus has practical complications as multiple incubators are required to maintain and manipulate these lines.

In future studies it will be of importance to fully characterise the transfection effects in terms of the cells ability to undergo osteogenic differentiation and to give rise to cells of other marrow stromal cell lineages in both the absence and presence of inducers. Various stage and lineage specific markers such as CBFA-1, a potential genetic marker of osteoblastic commitment and differentiation (Ducy *et al*, 1997), collagen type I, STRO-1, BSP, PTHR1 and OC could be measured as an indication of the osteogenic potential of the cells. It will be necessary to observe the proliferation rate and the total number of passages which the cells undergo as an indication of an increased cellular life-span, and to observe whether the cells return to a pretransformed state on the removal of cofactors. Alternatively the capacity of the cells to form bone *in vivo* could be investigated through implantation into ceramic carriers and placement into a recipient animal. This has the added advantage of providing a local environment that is more permissive and physiological and likely to give a more accurate impression of the cells potential to proliferate and differentiate *in vivo*. Identification of strains or lines capable of providing support for haematopoiesis could be evaluated in open-transplant assays such as described by Krebsbach *et al* (1997). In summary, these studies have validated the use of plasmids expressing SV40 large T antigen under the control of an inducible promoter to generate strains of BMSC from adult human bone marrow. Although at an early stage, it is anticipated that this work will lead to the development of marrow stromal cell lines applicable to the identification of the factors that regulate lineage commitment expansion and maturation and to the elucidation of the changes in gene expression that accompany these events.

Transfected cells do not grow indefinitely, however, despite the continued expression of SV40 large T Ag, and after a certain number of cell divisions the cells enter crisis (Price *et al*, 1994). In human cells, in contrast to rodent cells, immortalisation is inefficient and viral genes such as SV40 large T Ag are capable only of reliably extending the lifespan amounting to 20-60 population doublings post-transfection (Boulanger and Blair, 1991, Shay and Wright, 1991). During crisis, cell growth is balanced by cell death, as cells lose viability as they attempt to divide. Continued expression of SV40 large T Ag and at least one mutational event is required for cells to exit crisis and become immortal (Shay and Wright, 1989). To date, although transformed, the cells described in this study have not yet passed through crisis and are therefore not yet truly immortalised.

However, it is thought that the phenotype of transformed cells pre-crisis is more akin to that of normal cells (Spelsberg *et al*, 1995) and phenotypic characterisation should be carried out prior to crisis where possible. Post-crisis, however, it is suggested that immortalised cells continue to be reversibly controlled by SV40 large T Ag inactivation (Wright *et al*, 1989), and immortalisation does provides cells of reduced phenotypic variation, and of indefinite lifespan.

This methodology provides a useful tool to study the differentiation of mesenchymal cells. Transformation of a heterogeneous marrow cell population *in vitro* could result in immortalisation of progenitor cells at different levels of differentiation and maturity, including multipotential stem cells. Cloning a range of cell phenotypes could form a library of cells, and gene expression banks at various levels of osteogenic commitment and differentiation. Secondly, investigation of phenotypic and genotypic alterations during differentiation has become possible through transfection of plasmids which conditionally immortalise cells and are extracellularly inducible enabling regulation of proliferation rates and differentiation. Use of these plasmids would also allow investigation of factors that may influence commitment to, and differentiation through, the osteogenic lineage.

Ultimately, transformation of foetal stem cells will unveil the potential for understanding the derivation of mesenchymal cells in the body. This will also enable evaluation of switching between the various lineages, establishment of control over the differentiation pathway of the cells, and investigation of the effects of ageing in health and disease. In the near future it is of interest to immortalise a STRO-1$^-$/AP$^-$ population, or a non-adherent population, which may represent the most immature, and potentially multipotential, population in the mesenchymal lineage.

Chapter 8

Conclusions

It is now well established that bone marrow serves as a repository for osteoblast precursors in the adult. Currently there is considerable interest in these cells as: a) a target for therapeutic intervention *in vivo* in the prevention or treatment of osteoporosis, and b) as a source of autologous cells for tissue reconstruction or wound repair following their expansion *in vitro*. For either approach to become a reality there is a need to identify the factors that regulate the size and developmental potential of this precursor population, and to understand how their number and activity are influenced by gender and age. This work has focused on the use of *in vitro* culture of human marrow stromal cells to evaluate some of these issues.

At the outset of this work two important assumptions were made based largely on the pioneering work of Friedenstein (1980, 1990): a) that osteogenic precursors are present within the CFU-F population of human bone marrow, and b) that the expression of alkaline phosphatase by cells within a colony is indicative of the onset of osteogenic differentiation (Aronow *et al*, 1990, Owen *et al,* 1990). Over the course of these experiments, it became apparent that the technical ease with which cultures of marrow stromal cells can be established from adult human bone marrow belies the biological complexity of the model. One of the major limitations is the reliance on a purely functional readout i.e. colony formation, to assess the marrow content of CFU-F. It quickly became apparent that relatively small changes in conditions of culture could dramatically alter the CFE of marrow cell suspensions, indicating that for a given set of culture conditions only a subset of CFU-F are being detected using this approach. It also became apparent that colony forming potential persists in the non-adherent subpopulation of cells for several days post-explantation, whereas the results of previous investigation had suggested that essentially 100% of CFU-F will adhere within 24 hours of their explantation (Friedenstein, 1971, Castro-Malaspina *et al*, 1980). It is as yet unknown whether the clonogenic cells in the non-adherent subpopulation represent a different class of CFU-F, or simply cells earlier in the developmental hierarchy than CFU-F. There is an urgent need to distinguish between these possibilities and to determine their developmental potential, and to identify the factors that regulate their transition from the non-adherent to the adherent state.

An attempt was made to standardise the conditions of isolation and culture of bone marrow stromal cells and to optimise CFE, but this was unsuccessful due largely to the existence of substantial inter-donor variation which was not obviously attributable to the donor age or sex. Recent drug history may have been an important factor, and at an early stage donors receiving high dose glucocorticoid therapy were excluded. Towards the end of the investigation, it also became apparent patients with lung cancer have depressed colony forming potential (Chasseing *et al,* 1997), a factor that was not anticipated at the start of this investigation and one which must be considered in the future.

A first step towards circumventing some of the difficulties described would be to utilise immunochemical as well as functional criteria to assay CFU-F. A number of antibodies have been developed recently that appear to recognise primitive osteogenic precursors and/or CFU-F (STRO-1, HOP-26, MUC18). Ideally, these would be used in combination to assay 'potential' CFU-F in the original cell population and in the adherent and non-adherent subpopulations at different times post explantation. The observed number of colonies formed under any given set of culture conditions could then be expressed as a percent of the theoretical maximum calculated from the immunochemical data. In the experiments described this would have enabled a rational approach to the optimisation of isolation and/or culture conditions for CFU-F as it would then be possible to track the fate of the precursor cells.

It is evident from this investigation that there is still much to learn regarding the basic biology of the marrow stromal cell system. For example, it is currently unknown whether CFU-F numbers are the same or different at different skeletal sites, and this may influence the design of protocols for their harvest and/or *ex-vivo* expansion. There is also evidence that the behaviour of cells of the osteoblastic lineage at different sites does differ (Marie *et al*, 1992, Marie and De Vernejoul, 1993, Kasperk *et al*, 1995, Aerssens *et al*, 1997). In this context, it was noticed that during the course of the experiments described, despite the use of conditions known to support the differentiation of adipocytes, adipogenesis was never observed in rib-derived marrow stromal cell cultures, although it was observed in cultures derived from the femur or femoral head. This represents further, albeit circumstantial evidence that there exist real differences in the developmental potential of marrow stromal cells at different skeletal sites, and in future studies the experimental design should reflect this.

The results of this investigation provide additional evidence for an important influence of cells in the non-adherent population, presumably haematopoietic in origin, on the adhesion and proliferation of stromal precursors in the early stages of culture. In the literature both positive and negative influences have been reported, although the cells types responsible and the factors they produce remain poorly characterised. Although few antibodies are as yet available for identification and separation of stromal precursor cells, many are available for cells of the haematopoietic lineage, and have been used extensively to separate cells within this population. To evaluate the interactions between haematopoietic and CFU-F *in vitro,* and identify key cells types which affect the stromal cell component, colony forming experiments could be performed in which specific immune cell subsets could be selectively depleted from BMSC using antibodies directed against CD antigens. The validity of this approach has been demonstrated recently (Hurley *et al*, 1995, Waller *et al*, 1995, Rickard *et al*, 1996). Alternatively, in reconstitution experiments specific immune cell subsets could be

added back to cultures of highly enriched CFU-F populations to observe their effects, alone or in combination, on growth and differentiation.

Another contributing variable in the colony forming assays described here is the presence of serum in the culture medium. An increase in colony formation normally accompanies an increase in percent serum, although significant batch to batch variation has been reported to occur with foetal calf serum (Aronow *et al*, 1990, Tsuji *et al*, 1990). This was a problem that was taken into account at the outset of this work, and all experiments were conducted using serum from only two batches that were selected for their ability to support colony formation from isolates of human bone marrow. However, use of different sera makes the comparison of data generated in different laboratories at best difficult, and at worst sometimes of little real value. The use of serum-free medium would avoid this problem, but it is currently not possible to obtain complete osteogenic differentiation in the absence of serum. Some progress has been made in identifying serum-free conditions that will support the *ex-vivo* expansion of adult human marrow stromal cells (Gronthos and Simmons, 1995, Kuznetsov *et al*, 1997), but their developmental potential has not been reported.

For autologous marrow stromal cell therapy to become a reality, it will be necessary to identify factors that promote their expansion *ex-vivo* in the shortest possible time without altering their potential for osteogenic differentiation. The results of this investigation suggest that PDGF fulfils this criteria, and that it is effective in the absence and presence of Dx. Additional and more rigorous studies will be required to confirm this possibility, including a more detailed assessment of the resulting cell populations' potential for osteogenic differentiation *in vivo*.

It is apparent from this investigation that before further attempts are made to elucidate the cellular mechanisms responsible for the decline in osteoblast numbers in osteoporosis, it will be necessary to establish baseline parameters for the normal population in relation to the age, sex and hormonal status of the donors. Ideally this would be achieved using marrow obtained from a clinically relevant site (hip or spine) using both immunochemical and functional criteria to determine its content of CFU-F and their developmental potential. In addition, for each donor, whole body and regional estimates of bone mineral density should be obtained as well as urine and serum samples for the analysis of biochemical markers of bone turnover. Once this information had been accumulated it would be possible to conduct meaningful studies on the population dynamics of the marrow stromal system in osteoporosis and in response to therapy.

A major objective of future studies should be the determination of the precise number and hierarchy of marrow stromal cell lineages. This is important because a major contributing factor in the development of osteoporosis may be an imbalance between differentiation of osteoblasts and cells of other marrow stromal cell lineages, in particular adipocytes. It is in this regard that the conditionally immortalised marrow stromal cell strain will have their greatest utility. It will be necessary first to evaluate the developmental potential of the different strains *in vivo*. An attempt should then be made to clone from those strains with the greatest developmental potential, mono-, bi- or tri-potential cell lines. These would then be used to establish the interrelationships between cells of different marrow stromal cell lineages and the extent to which interchange can occur between them.

The utility of the conditionally immortalised lines would not be limited to the definition of the number and hierarchy of marrow stromal cell lineages. An additional application would be the generation of a panel of monoclonal antibodies recognising lineage and differentiation-stage specific cell surface antigens. These would prove invaluable in the diagnosis of skeletal disorders and in monitoring the response of cells of the marrow stromal system to therapy. They would also enable conditions of culture to be optimised for the *ex-vivo* expansion of cells of defined developmental potential and the enrichment of specific cell populations prior to re-implantation.

The conclusions drawn from this study indicate that an enormous amount of ground work is necessary to lead to a greater understanding of these cells and their development. The process of osteoblastic commitment, expansion and maturation remains highly complex and much research is necessary to elucidate the sequence of events leading to osteopenia, and ultimately to develop methods for treatment or prevention of osteoporosis.

In summary, the need to develop new therapeutic treatments for the prevention and treatment of osteoporosis and the potential of using *ex-vivo* expanded cells for tissue reconstruction and gene therapy make compelling arguments for continuing to study the stromal system of bone and marrow. Much work is still required, however, before these goals are realised.

Appendix I

Preparation of culture solutions and reagents

Cell culture

1 x DMEM (Dulbecco's Modified Eagle's medium (product # 52100-021) was prepared by mixing the following:

10 x DMEM (with non-essential amino acids)	100 ml
HEPES buffer	20 mM
sodium bicarbonate	0.09%
sodium pyruvate	0.1 mM
L-glutamine	200 mM
penicillin : streptomycin antibiotics mixture	25 IU/ml: 25 µg/l

This was made up to 1 litre with milli-Q water (Millipore Ltd., U.K.) and adjusted to pH 7.2 with fresh 5 M sodium hydroxide before it was sterilised by filtration. It was then aliquotted into 500 ml autoclaved bottles and stored at 4°C for a maximum of two weeks. All tissue culture stocks were supplied by Gibco BRL.

Foetal calf serum (FCS) supplement was obtained from Globepharm, U.K. (batch #2704) or Sigma Chemical Company (lot #105 H3350). Serum was heat inactivated at 56°C for 30 mins prior to use, sterilised by filtration and stored in 50 ml aliquots at -20°C.

A 10 mg/ml solution of L-ascorbic acid, sodium salt (Sigma) was prepared fresh in serum-free DMEM and filter sterilised.

A 10 mg/ml stock solution of L-ascorbic acid 2-phosphate (magnesium salt n-Hydrate, Wako Pure Chemical Industries Ltd.) was prepared in serum-free DMEM and filter sterilised. ASP was stored frozen in small aliquots at -20°C. Once thawed ASP was kept for up to two weeks at 4°C.

Phosphate buffered saline (calcium and magnesium free, Oxoid). Ten PBS tablets were dissolved in 1 litre of milli-Q water. The solution was adjusted to pH 7.2, autoclaved at 115°C for 10 mins and allowed to cool to room temperature before use.

10% (v/v) neutral buffered formalin (NBF) was made up by dissolving 100 ml formaldehyde, 16 g $Na_2HPO_4.12H_2O$ and 4 g $NaH_2PO_4.2H_2O$ in 1 litre of distilled water.

0.1 M Tris buffer was prepared by dissolving 12.1 g Tris in 1 litre of distilled water. The pH was adjusted to pH 8.2 with 5 M HCl.

5 mM inorganic phosphate (Pi) solution was prepared as a 0.01% (v/v) mixture of a 4:1 (v/v) ratio of 500 mM $Na_2HPO_4.12H_2O$: 500 mM $NaH_2PO_4.2H_2O$. The solution was adjusted to pH 7.4 with fresh 1 M NaOH and filtered.

A 0.1% (w/v) toluidine blue O solution was prepared in 30% (v/v) ethanol.

Borate buffer was prepared by titrating a 10 mM sodium borate solution, prepared in milli-Q water, with 10 mM boric acid to pH 8.8.

1% (w/v) methylene blue was prepared in 10 mM borate buffer, pH 8.8.

Protein Determination

Nonidet P-40 buffer was made up by dissolving 150 mM NaCl, 1% (v/v) NP-40 and 50 mM Tris in PBS. The solution was adjusted to pH 8.0 with fresh 1 M NaOH.

A 25 x stock protease inhibitor solution was prepared according to the manufacturers instructions (Complete™ Protease Inhibitor Cocktail Tablets, Boehringer Mannheim). One tablet was dissolved in 2 ml milli-Q water. 100 µl aliquots were stored at -20°C for up to 1 month.

20 g of sodium hydroxide pellets were dissolved in 50 ml milli-Q water to make a 10 M solution.

Growth factors

Serum-free medium was made up by adding 500 µg/ml BSA-linoleic acid (BLA), 200 ng/ml sodium selenite (NaS) and 5 µg/ml human apo-transferrin (APO-T) to serum-free DMEM.

1,25-dihydroxyvitamin D_3 (334 µM stock, gift from Roche, Welwyn Garden City) was stored at -70°C under an atmosphere of nitrogen. This was diluted 5 µl in 167 µl of DMEM, and added to serum-free medium at 1 in 1000 dilution (10^{-8} M final concentration).

Immunocytochemistry

FACS wash buffer was prepared with 10 mM HEPES and 5% FCS in Hanks Balanced salt solution adjusted to pH 7.35 with 1 M HCl.

Blocking buffer: 10% (v/v) normal human serum and 1% (w/v) BSA were diluted and dissolved in FACS wash buffer.

FACS fix was prepared as 2% (v/v) paraformaldehyde in FACS wash buffer.

0.1% (w/v) saponin was dissolved in FACS wash buffer.

Western Blot solutions

10 x Tank buffer (pH 8.3) was prepared with the following reagents in milli-Q water:

Tris-Cl	0.25 M
glycine	1.92 M
SDS	1%

2 x Protein sample loading buffer was prepared with the following reagents in milli-Q water and adjusted to pH 6.8. It was stored in 0.5 ml aliquots at -20°C for up to 6 months.

4 x 0.5 M Tris-Cl (pH 6.8)	0.125 M
10% SDS	4% (v/v)
glycerol	20% (v/v)
bromophenol blue	0.02%
Dithiothreitol	0.2 M

Towbin Transfer buffer was prepared with the following reagents in milli-Q water and adjusted to pH 8.3.

Tris-Cl	25 mM
glycine	192 mM
SDS	0.1% (v/v)
methanol	20% (v/v)

0.025% (w/v) Coomassie Brilliant blue R 250 was dissolved in 40% (v/v) methanol and 7% (v/v) acetic acid in milli-Q water.

Destaining solution was 40% (v/v) methanol and 7% (v/v) acetic acid in milli-Q water.

Preparation of cDNA

The contents of the mRNA extraction kit (QuickPrep® Micro mRNA Purification kit, Pharmacia Biotech) included:

Oligo(dT)-cellulose	25 mg/ml suspended in a storage buffer containing 0.15% Kathon ® CG.
Extraction buffer	Buffered aqueous solution of guanidium thiocyanate and N-lauroyl sarcosine.
High salt buffer	10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 M NaCl
Low salt buffer	10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M NaCl
Glycogen solution	Glycogen at 5-10 mg/ml in DEPC-treated water.
Potassium acetate	2.5 M potassium acetate (pH 5.0)
Elution buffer	10 mM Tris-HCl (pH 7.5), 1 mM EDTA

DEPC-treated water was prepared as a 0.1% (v/v) solution of diethyl pyrocarbonate (DEPC) in distilled water. It was allowed to stand overnight at room temperature and then autoclaved, and allowed to cool before use.

Wash buffer was 1% (v/v) Tween-20 in PBS.

20 x SSC was prepared by dissolving 175.3g NaCl and 88.2g Na Citrate in 800 ml milli-Q water, adjusted to pH 7.0, then made up to 1 litre and autoclaved.

Blotto was prepared with 5% (w/v) non-fat milk powder, 1% (w/v) goat serum and 1% (v/v) Tween-20 in PBS.

Prehybridisation solution was a mixture of 6 x SSC and 0.05% (v/v) Blotto in milli-Q water.

Hybridisation buffer was a mixture of 6 x SSC and 0.5 % (w/v) SDS in milli-Q water.

DNA methods

The contents of the DNA isolation kit included:

Cell Resuspension solution	50 mM Tris-HCl, pH 7.5
	10 mM EDTA
	100 μg/ml RNase
Cell Lysis solution	2 mM NaOH
	0.1% SDS
Neutralisation solution (pH 4.8)	2.55 M Potassium Acetate
TE buffer (pH 7.3)	10 mM Tris-HCl
	1 mM EDTA
Column Wash solution (pH 7.5)	200 mM NaCl
	20 mM Tris-HCl
	5 mM EDTA
	Diluted 1:1 with 95% ethanol prior to use.

10 x TAE buffer (Tris-acetate final concentration was 0.04 M, pH 8.3) was prepared by mixing 48 g Tris base and 11.42 ml Glacial acetic acid in 20 ml 0.5 M EDTA (pH 8.0) solution:

5 x DNA loading dye was prepared with the following reagents in milli-Q water:

0.75 M Na_2EDTA (pH 8.3)	0.2 M
SDS	10%
glycerol	50%
bromophenol blue	0.2%

Transfection methods

HEPES-buffered saline (HBS) was prepared with the following reagents in milli-Q water:

HEPES	20 mM
NaCl	137 mM
Glucose	6 mM
KCl	5 mM
$Na_2H_2PO_4$	0.7 mM

This solution was then sterilised through a 22 μm filter.

Solutions used for β-galactosidase staining were:

Phosphate buffered Saline (pH 7.3)	Sodium Chloride	150 mM
	Sodium Phosphate	15 mM
Fixative Solution	Glutaraldehyde	1%
	$MgCl_2$	1 mM
	Sodium Phosphate	0.1M
X-Gal Solution	X-Gal	0.2%
	$MgCl_2$	1 mM
	NaCl	150 mM
	$K_4Fe(CN)_6$	3.3 mM
	$K_3Fe(CN)_6$	3.3 mM

Appendix II

Statistical analyses of ageing data

The following tables of Spearmans rank correlation coefficient analyses indicate the influence of age on BMSC colony formation and proliferation. The data for these analyses are described in chapter 6, and the graph to which the table relates is stated in parentheses.

Analyses of CFE

Analysis 1.1 Donor age vs total (i) and AP (ii) colony formation - Dx (figure 6.2)

i)

Sum of Squared Differences	19091.500
Rho	.136
Z-Value	.963
P-Value	.3358
n	51
critical value (a1)	.2329

ii)

Sum of Squared Differences	7188.000
Rho	.213
Z-Value	1.299
P-Value	.1941
n	38
critical value (a1)	.2710

Analysis 1.2 Donor age vs total (i) and AP (ii) colony formation + Dx (figure 6.2)

i)

Sum of Squared Differences	12084.500
Rho	.088
Z-Value	.567
P-Value	.5705
n	43
critical value (a1)	.2543

ii)

Sum of Squared Differences	6400.000
Rho	.176
Z-Value	1.043
P-Value	.2969
n	36
critical value (a1)	.2788

Analysis 2.1 Male (i) and female (ii) donor ages vs total colony formation - Dx (figure 6.3)

i)

Sum of Squared Differences	4629.500
Rho	.293
Z-Value	1.681
P-Value	.0927
n	34
critical value (a1)	.2871

ii)

Sum of Squared Differences	1428.000
Rho	.194
Z-Value	.888
P-Value	.3748
n	22
Critical value (a2)	.4252

Analysis 2.2 **Male (i) and female (ii) donor ages vs AP+ colony formation in - Dx (figure 6.3)**

i)

Sum of Squared Differences	814.000
Rho	.002
Z-Value	.010
P-Value	.9922
n	17
Critical value (a2)	.4877

ii)

Sum of Squared Differences	420.500
Rho	.382
Z-Value	1.478
P-Value	.1394
Rho corrected for ties	16
Tied Z-Value	.5029

Analysis 2.3 **Male (i) and female (ii) donor age ≥ 50 years vs total colony formation - Dx (figure 6.3)**

i)

Sum of Squared Differences	3252.50
Rho	.28
Z-Value	1.49
P-Value	.1366
n	30
Critical value (a2)	.3624

ii)

Sum of Squared Differences	440.00
Rho	.21
Z-Value	.80
P-Value	.4227
n	15
Critical value (a2)	.5214

Analysis 2.4 **Male (i) and female (ii) donor age ≥ 50 years vs AP+ colony formation - Dx (figure 6.3)**

i)

Sum of Squared Differences	1318.50
Rho	.01
Z-Value	.04
P-Value	.9699
n	20
Critical value (a2)	.4466

ii)

Sum of Squared Differences	326.50
Rho	.42
Z-Value	1.56
P-Value	.1187
n	15
Critical value (a2)	.5214

Analysis 3.1 **Male (i) and female (ii) donor ages vs total colony formation + Dx (figure 6.4)**

i)

Sum of Squared Differences	2718.000
Rho	.256
Z-Value	1.331
P-Value	.1832
n	28
critical value (a2)	.3755

ii)

Sum of Squared Differences	602.000
Rho	-.075
Z-Value	-.281
P-Value	.7790
n	15
critical value (a2)	.5214

Analysis 3.2 **Male (i) and female (ii) donor ages vs AP+ colony formation + Dx (figure 6.4)**

i)

Sum of Squared Differences	1333.500
Rho	.134
Z-Value	.600
P-Value	.5487
n	21
Critical value (a2)	.4364

ii)

Sum of Squared Differences	426.000
Rho	.239
Z-Value	.895
P-Value	.3706
n	15
Critical value (a2)	.5214

Analysis 3.3 **Male (i) and female (ii) donor age ≥ 50 years vs total colony formation + Dx (figure 6.4)**

i)

Sum of Squared Differences	1951.500
Rho	.152
Z-Value	.727
P-Value	.4674
n	24
Critical value (a2)	.4070

ii)

Sum of Squared Differences	431.000
Rho	.053
Z-Value	.190
P-Value	.8492
n	14
Critical value (a2)	.5385

Analysis 3.4 **Male (i) and female (ii) donor age ≥ 50 years vs AP+ colony formation + Dx (figure 6.4)**

i)

Sum of Squared Differences	1258.000
Rho	-.104
Z-Value	-.439
P-Value	.6606
n	19
Critical value (a1)	.3912

ii)

Sum of Squared Differences	276.000
Rho	.393
Z-Value	1.418
P-Value	.1561
n	14
critical value (a2)	.5385

Analyses of proliferation

Analysis 4.1 **Ages of all donors vs BMSC proliferation treated - (i) or + (ii) Dx (figure 6.5)**

i)

Sum of Squared Differences	4968.500
Rho	-.105
Z-Value	-.567
P-Value	.5705
n	30
critical value (a2)	.3624

ii)

Sum of Squared Differences	5033.000
Rho	-.240
Z-Value	-1.268
P-Value	.2047
n	29
critical value (a2)	.3685

Analysis 5.1 **Comparison of male (i) and female (ii) donor age with cell proliferation -Dx (figure 6.6)**

i)

Sum of Squared Differences	1650.000
Rho	-.241
Z-Value	-1.049
P-Value	.2943
n	20
critcal value (a2)	.4466

ii)

Sum of Squared Differences	230.000
Rho	-.394
Z-Value	-1.182
P-Value	.2373
n	10
critical value (a1)	.5636

Analysis 5.2 **Comparison of male (i) and female (ii) donor age with proliferation + Dx (figure 6.6)**

i)

Sum of Squared Differences	1974.500
Rho	-.282
Z-Value	-1.262
P-Value	.2070
n	21
critical value (a2)	.4364

ii)

Sum of Squared Differences	184.000
Rho	-.533
Z-Value	-1.508
P-Value	.1314
n	9
critical value (a1)	.6000

Analysis 5.3 **Male (i) and female (ii) donor ages ≥ 50 years vs cell proliferation -Dx(figure 6.6)**

i)

Sum of Squared Differences	737.500
Rho	-.085
Z-Value	-.327
P-Value	.7433
n	16
critical value (a2)	.5029

ii)

Sum of Squared Differences	86.000
Rho	-.536
Z-Value	-1.312
P-Value	.1894
n	7
critical value (a1)	.7143

Analysis 5.4 **Male (i) and female (ii) donor ages ≥ 50 years vs cell proliferation + Dx (figure 6.6)**

i)

Sum of Squared Differences	1323.000
Rho	-.365
Z-Value	-1.506
P-Value	.1320
n	18
critical value (a2)	.4716

ii)

Sum of Squared Differences	40.000
Rho	-.143
Z-Value	-.319
P-Value	.7494
n	6
critical value (a2)	.8857

The following tables show results of Mann-Whitney U comparisons and demonstrate the influence of donor gender (+/- Dx) independent of the influence of age on colony formation and proliferation. The data for these analyses are described in chapter 6, and the graph to which the table relates is stated in parentheses. Comparisons resulting in significant differences where $p < 0.0500$ have been italicised.

Analysis 16 **The effect of Dx treatment in a cohort study of total and AP colony formation (figure 6.7)**

Comparison	U value	P value
1 vs 2	935.5	.2217
3 vs 4	566.0	.2019

Analysis 17 **The influence of gender and Dx treatment on total and AP colony formation (figure 6.8i)**

Comparison	U value	P value
1 vs 2	399.0	.2761
3 vs 4	194.5	.3752
5 vs 6	116.0	.6641
7 vs 8	95.0	.3230
1 vs 5	*181.5*	*.0317*
2 vs 6	152.0	.1429
3 vs 7	153.5	.5059
4 vs 8	146.0	.7121

Analysis 18 **The influence of gender and Dx treatment on total and AP colony formation of donors of ≥ 50 years (figure 6.8ii)**

Comparison	U value	P value
1 vs 2	312.5	.4083
3 vs 4	159.5	.3915
5 vs 6	129.0	.8009
7 vs 8	117.5	.5052
1 vs 5	*117.0*	*.0022*
2 vs 6	*100.0*	*.0111*
3 vs 7	126.5	.1849
4 vs 8	113.5	.2024

Analysis 19 **The effect of Dx treatment in a cohort study of BMSC proliferation (figure 6.9)**

Comparison	U value	P value
1 vs 2	367	.276

Analysis 20 **The influence of gender and Dx treatment on BMSC proliferation (figure 6.10i)**

Comparison	U value	P value
1 vs 2	152	.1941
3 vs 4	33.0	.5340
1 vs 3	***52.0***	***.0347***
2 vs 4	46.5	.0884

Analysis 21 **The influence of gender and Dx treatment on proliferation of BMSC from donors of ≥ 50 years (figure 6.10ii)**

Comparison	U value	P value
1 vs 2	104	.2490
3 vs 4	15	.3914
1 vs 3	***19.0***	***.0134***
2 vs 4	***19.5***	***.0274***

Appendix III

Donor details

The following table summarises the available characteristics of the donors used in this study. All samples are rib unless stated otherwise. U, unknown.

Donor #	Sex	Age (yrs)	Diagnosis	Drugs
343	F	77	U	U
347	M	55	U	U
348	F	56	U	U
352	F	57	U	U
353	M	63	U	U
356	M	27	U	U
361	M	74	U	U
362	M	74	U	U
367	M	56	U	U
372	M	74	U	U
374	F	84	U	U
375	M	71	U	U
386	M	66	U	U
387	F	71	U	U
388	M	61	U	U
393	F	26	U	U
395	F	73	U	Thyroxine Aspirin Amilodipine
399	M	66	U	U
409	F	39	U	U
418	M	75	osteoarthritis	Nifedipine Naprosyn
422	M	62	lung carcinoma	Ventoline Becotide
428	M	55	U	U
429	M	54	lung carcinoma	U
434	M	73	U	U
439	M	66	U	Heparin Cefuroxine
441	M	66	U	Omerplazole Amilodipine

Donor #	Sex	Age (yrs)	Diagnosis	Drugs
443	M	63	lung carcinoma	Predinisolone Ventolin Attrovent Metzlerzone
448	M	79	oesophagitis	Losec Laptropril Ferrous Sulphide Bactofen Ranitidine
464	M	58	small cell carcinoma	Naproxam Lansoprazole
466	M	74	lung carcinoma	Elantan Attenolol GTN Spray Aspirin Bendrofluazide
468	F	36	spinal Fusion	U
472	M	67	U	U
475	F	60	lung carcinoma	U
480	M	80	lung carcinoma	Dothiapan
481	F		U	U
482	M	U	squamous cell carcinoma	Innovace Bendrofluazide
486 (Femur)	F	84	rheumatoid arthritis	Voltarol Coproximal Dihydrocodeine Temazapam
491	F	55	hiatus hernia	Lansoprazole Metaclopramide
496	F	74	U	Framil
500	F	70	bronchial carcinoma	Nifedipine Dothiepin Bendrofluazide
507	F	72	U	U
518	M	54	6th rib	Propranol

Donor #	Sex	Age (yrs)	Diagnosis	Drugs
520	F	76	lung carcinoma	U
523	F	62	lung carcinoma	Zantac Clomipramin
526	F	63	lung carcinoma	Amilodipine Naproxen
527	F	42	achalasia	U
530	M	52	lung carcinoma	U
533	F	67	carcinoma of lower oesophagus	Bisoprolol Loloclamol Nifedipine Maxolon Pettidium
539	M	71	U	U
546	M	15	neurofibratosis	Tamazepam Heparin Metoclopramide Cefuroxamine
550	M	75	oesophago-gastrectomy	U
552	F	73	acimyosarcoma of uterus	U
562	M	71	lung carcinoma	U
571	F	58	lung carcinoma	Tendnormlin LS
572	F	61	lung carcinoma	Paracetamol Tamazepam Hapazine Cefaxoxime
573	M	71	stomach carcinoma	U

Bibliography

Adams, J.C., Watt, F.M. (1993) Regulation of development and differentiation by the extracellular matrix. *J. Cell Sci.* **117** 1183-1198

Adinoff, A.D., Hollister, J.R. (1983) Steroid-induced fractures and bone loss in patients with asthma. *N. Engl. J. Med.* **309** 265-268

Aerssens, J., Boonen, S., Joly, J., Dequeker, J. (1997) Variations in trabecular bone composition with anatomical site and age: potential implications for bone quality assessment. *J. Endo.* **155** 411-421

Andreason, G.L., Evans, G.A. (1988) Introduction and expression of DNA molecules in eukaryotic cells by electroporation. *BioTechniques* **6** 650-660

Andrew, J. G., Hoyland, J. A., Freemont, A. J., Marsh, D.R. (1995) Platelet-derived growth factor expression in normally healing human fractures. *Bone* **16** 455-460

Archer, C.W., Cottrill, C.P., Lee, D. (1990) Effects of ascorbate on myogenesis in micromass culture. *In vitro Cell Dev. Biol.* **26** 259-264

Aronow, M.A., Gerstenfeld, L.C., Owen, T.A., Tassinari, M.S., Stein, G.S., Lian, J.B. (1990) Factors that promote progressive development of the osteoblast phenotype in cultured fetal rat calvarial cells. *J. Cell. Phys.* **143** 213-221

Arts, J., Kuiper, G.G., Janssen, J.M., Gustafsson, J.A., Lowik, C.W., Pols, H.A., van Leeuwen, J.P. (1997) Differential expression of estrogen receptors alpha and beta mRNA during differentiation of human osteoblast SV-HFO cells. *Endo.* **138** 5067-5070

Aubin, J.E., Fung, S.W., Georgis, W. (1990) The influence of non-osteogenic hemopoietic cells on bone formation by bone marrow stromal populations. *J. Bone Min. Res.* **5** S81

Aubin, J.E., Turksen, K., Heershe, J.N.M. (1993) Osteoblastic cell lineage. In: Cellular and molecular biology of the bone. ed. Noda, M. Academic Press, San Diego. 1-45

Aubin, J.E., Gupta, A.K., Bhargava, U., Turksen, K. (1996) Expression and regulation of galectin 3 in rat osteoblastic cells. *J. Cell. Physiol.* **169** 486-480

Bab, I., Ashton, B.A., Syftetad, G.T., Owen, M.E. (1984) Assessment of an *in vivo* diffusion chamber method as a quantitative assay for osteogenesis. *Calcif. Tiss. Int.* **36** 77-82

Banerjee, C., Hiebert, SW., Stein J.L., Lian, J.B., Stein, G.S. (1995) An AML-1 consensus sequence binds an osteoblast-specific complex and transcriptionally activates the osteocalcin gene. *Proc. Natl. Acad. Sci. USA.* **93** 4968-4973

Banerjee, C., McCabe, L.R., Choi, J.Y., Hiebert, S.W., Stein, J.L., Stein, G.S., Lian, J.B. (1997) Runt homology domain proteins in osteoblasts differentiation: AML3/CBFA1 is a major component of a bone-specific complex. *J. Cell. Biochem.* **66** 1-8

Bronckers, A.L., Gay, S., Finkelman, R.D., Butler, W.T. (1987) Developmental appearence of Gla proteins (osteocalcin) and alkaline phosphatase in tooth germs and bones of the rat. *Bone and Miner.* **2** 361-373

Baron, R. (1993) Anatomy and ultrastructure of bone. In: Primer on the metabolic bone diseases and disorders of bone and mineral metabolism. Raven Press, New York 3-9

Beardsworth, L.J., Eyre, D.R., Dickson, I.R. (1990) Changes with age in the urinary excretion of lysyl- and hydroxylysylpyridinoline, two new markers of bone collagen turnover. *J. Bone Min. Res.* **5** 671-676

Behr, J.P., Demeneix, B., Loeffler, J.P., Perez-Mutul, J. (1989) Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc. Natl. Acad. Sci. USA.* **86** 6982-6986

Bellows, C.G., Aubin, J.E. (1989) Determination of numbers of osteoprogenitors present in fetal rat calvarial cells *in vitro. Dev. Biol.* **133** 8-13

Bellows, C.G., Heersche, J.N.M., Aubin, J.E. (1990) Determination of the capacity for proliferation and differentiation of osteoprogenitor cells in the presence and absence of dexamethasone. *Dev. Biol.* **140** 132-138

Bellows, C.G., Ciaccia, A., Heersche, J.N.M. (1998) Osteoprogenitor cells in cell populations derived from mouse and rat calvaria differ in their response to corticosterone, cortisol, and cortisone. *Bone* **23** 119-125

Benayahu, D., Efrati, M., Weintroub, S. (1995) Monoclonal antibodies recognise antigen expressed by osteoblasts. *J. Bone Min. Res.* **10** 1496-1503

Benayahu, D., Kletter, Y., Zipori, D., Weintraub, S. (1989) Bone marrow-derived stromal cell line expressing osteoblastic phenotype *in vitro* and osteogenic capacity *in vivo. J. Cell. Physiol.* **140** 1-7

Beresford, J.N. (1989) Osteogenic stem cells and the stromal system of bone and marrow. *Clin. Orthop.* **240** 270-280

Beresford, J.N., Bennet, J.H., Devlin, C., Leboy, P.S., Owen, M.E. (1992) Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. *J. Cell Sci.* **102** 341-351

Beresford, J.N., Graves, S.E., Smoothy, C.A. (1993) Formation of mineralized nodules by bone-derived cells *in vitro*: a model of bone formation? *Am. J. Med. Genet.* **45** 163-178

Beresford, J.N., Joyner, C.J., Devlin, C., Triffit, J.T. (1994) The effects of dexamethasone and 1,25-dihydroxyvitamin D_3 on osteogenic differentiation of human marrow stromal cells *in vitro*. *Archs. Oral Biol.* **39** 941-947

Bertolini, D.R., Nedwin, G.E., Bringman, T.S., Smith, D.D., Mundy, G.R. (1986) Stimulation of bone resorption and inhibition of bone formation *in vitro* by human tumour necrosis factors. *Nature* **319** 516-518

Bianco, P., Silvestrini, G., Termine, J.D., Bonucci, E. (1988) Immunohistochemical localisation of osteonectin in developing human and calf bone using monoclonal antibodies. *Calcif. Tiss. Int.* **43** 155-161

Bianco, P., Fisher, L.W., Young, M.F., Termine, J.D., Gehron-Robey, P. (1991) Expression of bone sialoprotein (BSP) in human developing skeletal and nonskeletal tissues as revealed by immunostaining and *in situ* hybridization. *Calcif Tiss. Int.* **49** 421-426

Bianco, P., Riminucci, M., Bonucci, E., Termine, J.D., Robey, P.G. (1993) Bone sialoprotein (BSP) secretion and osteoblast differentiation - relationship to bromodeoxyuridine incorporation, alkaline phosphatase, and matrix deposition. *J. Histochem. Cytochem.* **41** 183-191

Bianco, P., Riminucci, M. (1998) The bone marrow stroma *in vivo*: ontogeny, structure, cellular composition and changes in disease. In: Marrow stromal cell culture. eds. Beresford, J.N., Owen, M. Cambridge University Press. 10-25

Bilezikian, J.P., Raisz, L.G., Rodan, G.A. eds. (1996) Principles of bone biology. Academic Press. 305-729

Bikle, D.D., Halloran, B., Fong, L., Steinbach, L., Shellito, J. (1993) Elevated 1,25-dihydroxyvitamin D levels in patients with chronic obstructive pulmonary disease treated with prednisone. *J. Clin. Endocrinol. Metab.* **76** 456-461

Bodine, P.V.N., Henderson, R.A., Green, J., Aronow, M., Owen, T., Stein, G.S., Lian, J.B., Komm, B.S. (1998) Estrogen receptor-alpha is developmentally regulated

during osteoblast differentiation and contributes to selective responsiveness of gene expression. *Endo.* **139** 2048-2057

Bodine, P.V.N., Trailsmith, M., Komm, B.S. (1996) Development and characterization of a conditionally transformed adult human osteoblastic cell line. *J. Bone Min. Res.* **11** 806-819

Bonjour, J.P., Theintz, G., Buchs, B., Slosman, D., Rizzoli, R. (1991) Critical years and stages of puberty for spinal and femoral bone mass accumulation during adolescence. *J. Clin. Endo. Metab* **73** 555-563

Bouillon, R., Bex, M., Van Herck, E., Laureys, J., Dooms, L., Lesaffre, E., Ravussin, E. (1995) Influence of age, sex and insulin on osteoblast function: osteoblast dysfunction in diabetes mellitus. *J. Clin. Endo. Metab.* **80** 1194-1202

Boulanger, P.A., Blair, G.E. (1991) Expression and interactions of human adenovirus oncoproteins. *Biochem. J.* **275** 281-299

Bruder, S.P., Caplan, A.I. (1989) First bone formation and the dissection of an osteogenic lineage in the embryonic chick tibia is revealed by monoclonal antibodies against osteoblasts. *Bone* **10** 359-375

Bruder, S.P., Caplan, A.I. (1990) A monoclonal antibody against the surface of osteoblasts recognises alkaline phosphatase isoenzymes in bone, liver, kidney, and intestine. *Bone* **11** 133-139

Bruder, S.P., Caplan, A.I. (1990a) Terminal differentiation of osteogenic cells in the embryonic chick tibia is revealed by a monoclonal antibody against osteocytes. *Bone* **11** 189-198

Bruder, A.P., Horowitz, M.C., Mosca, J.D., Haynesworth, S.E. (1997) Monoclonal antibodies reactive with human osteogenic cell surface antigens. *Bone* **21** 225-235

Bruder, S.P., Ricalton, N.S., Boynton, R.E., Conolly, T.J., Jaiswal, N., Zala, J., Barry, F.P. (1998) Mesenchymal stem cell surface antigen SB-10 corresponds to activated leukocyte cell adhesion molecule and is involved in osteogenic diffenrtion. *J. Bone Min. Res.* **13** 655-663

Bryckaert, M.C., Lindroth, M., Lonn, A., Tobelem, G., Wasteson, A. (1988) Transforming growth factor (TGFβ) decreases the proliferation of human bone marrow fibroblasts by inhibiting the platelet-derived growth factor (PDGF) binding. *Exp. Cell Res.* **179** 311-321

Burkhardt, R., Kettner, G., Bohm, W., Schmidmeir, M., Schlag, R., Frisch, B., Mallmann, B., Eisenmenger, W., Gilg, T.H. (1987) Changes in trabecular bone, hematopoesis and bone marrow vessels in aplastic anaemia, primary osteoporosis and old age: A comparative histomorphometric study. *Bone* **8** 157-164

Canalis, E. (1987) Effects of tumour necrosis factor on bone formation *in vitro*. *Endo.* **121** 1596-1604

Canalis, E., McCarthy, T.L., Centrella, M. (1989) Effects of platelet-derived growth factor on bone formation *in vitro*. *J. Cell. Physiol.* **140** 530-537

Cassiede, P., Dennis, J.E., Ma, F., Caplan, A.I. (1996) Osteochondrogenic potential of marrow mesenchymal progenitor cells exposed to TGF-β1 or PDGF-BB as assayed *in vivo* and *in vitro*. *J. Bone Min. Res.* **11** 1264-1273

Castro-Malaspina, H., Gay, R.E., Resnick, G., Kapoor. N., Meyers, P., Chiarieri, D., McKenzie, S., Broxmeyer, H.E., Moore, M.A.S. (1980) Characterisation of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. *Blood* **56** 289-301

Castro-Malaspina, H., Rabellino, E.M., Yen, A., Nachman, R.L., Moore, M.A. (1981) Human megakaryocyte stimulation of proliferation of bone marrow fibroblasts. *Blood* **57** 781-787

Centrella, M., McCarthy, T.L., Canalis, E. (1989) Platelet-derived growth factor enhances deoxyribonucleic acid and collagen synthesis in osteoblast-enriched cultures from fetal rat parietal bone. *Endo.* **125** 13-19

Centrella, M., McCarthy, T.L., Kusmik, W.F., Canalis, E. (1991) Relative binding and biochemical effects of heterodimeric and homodimeric isoforms of platelet-derived growth factor in osteoblast-enriched cultures from fetal rat bone. *J. Cell. Phys.* **147** 420-426

Centrella, M., McCarthy, T.L., Canalis, E. (1991a) Glucocorticoid regulation of transforming growth factor β1 activity and binding in osteoblast-enriched cultures from fetal rat bone. *Mol. Cell. Biol.* **11** 4490-4496

Centrella, M., McCarthy, T.L., Kusmik, W.F., Canalis, E. (1992) Isoform-specific regulation of platelet-derived growth factor activity and binding in osteoblast-enriched cultures from fetal rat bone. *J. Clin. Invest.* **89** 1076-1084

Chasseing, N.A., Bordenave, R.H., Bullorsky, E.O., Diaz, N.B., Stemmelin, G.R., Rumi, L.S. (1997) Fibroblastic colony-formation units and levels of tumor necrosis

factor and prostaglandin E_2 in bone marrow cultures from patients with advanced lung carcinoma. *Cancer* **80** 1914-1919

Chasseing, N.A., Trejo, Y.G., Bordenave, R.H., Bullorsky, E.O., Diaz, N.B., Rumi, L.S. (1997a) Bone marrow fibroblastic progenitors in patients with advanced breast cancer. *Breast Cancer Res. and Treatment* **45** 211-218

Chawla, A., Schwarz,E.J., Dimaculangan, D.D., Lazar, M.A. (1994) Peroxisome proliferator-activated receptor (PPAR) gamma: adipose-predominant expression and induction early in adipocyte differentiation. *Endo.* **135** 798-800

Chen, T.L., Mallory ,J.B., Hintz, R.L. (1991) Dexamethasone and 1,25 $(OH)_2$ vitamin D_3 modulate the synthesis of insulin-like growth factor-I in osteoblast-like cells. *Calcif. Tiss. Int.* **48** 278-282

Chen, T.L., Cone, C.M., Feldman, D. (1983) Glucocorticoid modulation of cell proliferation in cultured osteoblastic-like bone cells: differences between rat and mouse. *Endo.* **112** 1739-1744

Chen, T.L., Feldman, D. (1979) Glucocorticoid receptors and actions in subpopulations of cultured rat bone cells: mechanism of dexamethasone potentiation of parathyroid hormone-stimulated cyclic AMP production. *J. Clin. Invest.* **63** 650-785

Cheng, S.L., Shen, V. Peck, W.A. (1991) Regulation of plasminogen activator and plasminogen activator inhibitors production by growth factors and cytokines in rat calvarial cells. *Calcif. Tiss. Int.* **49** 321-327

Cheng, S.L., Yang, J.W., Rifas, L., Zhang, S.F., Avioli, L.V. (1994) Differentiation of human bone marrow osteogenic stromal cells *in vitro*: Induction of the osteoblast phenotype by dexamethasone. *Endo.* **134** 277-286

Cheng, S.L., Zhang, S.F., Avioli L.V. (1996) Expression of bone matrix proteins during dexamethasone-induced mineralisation of human bone marrow stromal cells. *J. Cellular Biochem.* **61** 182-193

Chenu, C., Pfeilschifter, J., Mundy, G.R., Roodman, G.D. (1988) Transforming growth factor-β inhibits formation of osteoclast-like cells in long term human marrow cultures. *Proc Natl Acad Sci USA* **85** 5683-5687

Choi, J.Y., Lee, B.H., Song, K.B., Park, R.W., Kim, I.S., Sohn, K.Y., Jo, O.S., Ryoo, H.M. (1996) Expression patterns of bone-related proteins during osteoblastic differentiation in MC3T3-E1 cells. *J. Cellular Biochem.* **61** 609-618

Chow, J., Chambers, T.J. (1992) An assessment of the prevalence of organic material on bone surfaces. *Calcif. Tiss. Int.* **50** 118-122

Cicuttini, F.M., Martin, M., Salvaris, E., Ashman, L., Begley, C.G., Novotny, J., Maher, D., Boyd, A.W. (1992) Support of human cord blood progenitor cells on human stromal cell lines transformed by SV_{40} large T antigen under the influence of an inducible (metallothionein) promoter. *Blood* **80** 102-112

Cochran, D.L., Rouse, C.A., Lynch, S.E. Graves DT. (1993) Effects of platelet-derived growth factor isoforms on calcium release from neonatal mouse calvariae. *Bone* **14** 52-58

Compston, J.E., Vedi, S., Mellish, R.W.,E., Croucher, P.E., O'Sullivan, M.M. (1989) Reduced bone formation in non-steroid treated patients with rheumatoid arthritis. *Ann. Rheum. Dis.* **48** 483-487

Compston, J.E., Croucher, P.I. (1991) Histomorphometric assessment of trabecular bone remodelling in osteoporosis. *Bone and Mineral* **14** 91-102

Croisille, L., Auffray, I., Katz, A., Izac, B., Vainchenker, W., Coulombel, L. (1994) Hydrocortisone differentially affected the ability of murine stromal cells and human marrow-derived adherent cells to promote the differentiation of $CD34^{++}/CD38^{-}$ long-term culture-initiating cells. *Blood* **84** 4116-4124

Currie, G.A. (1981) Platelet-derived growth factor requirements for *in vitro* proliferation of normal and malignant cells. *Br. J. Cancer* **43** 335-343

d'Avis, P.Y., Frazier, C.R., Shapiro, J.R., Fedarko, N.S. (1997) Age-related changes in effects of insulin-like growth factor I on human osteoblast-like cells. *Biochem. J.* **324** 753-760

Darnell, J., Lodish, H., Baltimore, D. (1986) Molecular cell biology. American Books, U.S.A. 1042-1047

Davis, J.W., Ross, P.D., Wasnich, R.D. (1994) Evidence for both generalised and regional low bone mass among elderly women. *J. Bone Min. Res.* **9** 305-309

Davis, S., Aldrich, T.H., Ip, N.Y., Stahl, N., Scherer, S., Farrugella, T., DiStefano, P.S., Curtis, R., Panayotatos, N., Gascan, H., Chevalier, S., Yancopoulos, G.D. (1993) Released form of CNTF receptor α component as a soluble mediator of CNTF responses. *Science* **259** 1736-1739

DeAngelis, T., Ferber, A., Baserga, R. (1995) Insulin-like growth factor I receptor is required for the mitogenic and transforming activities of the platelet-derived growth factor receptor. *J. Cell. Phys.* **164** 214-221

Defranco, D.J., Lian, J.B., Glowaki, J. (1992) Differential effects of glucocorticoid on recruitment and activity of osteoclasts induced by normal and osteocalcin-deficient bone implanted in rats. *Endo.* **131** 114-121

Delany, A.M., Pash, J.M., Canalis, E. (1994) Cellular and clinical perspectives on skeletal insulin-like growth factor I. *J. Cellular Biochem.* **55** 328-333

Delany, A.M., Dong, Y., Canalis, E. (1994a) Mechanisms of glucocorticoid action in bone cells. *J. Cellular Biochem.* **56** 295-302

dePollak, C., Arnaud, E., Renier, D., Marie, P.J. (1997) Age-related changes in bone formation, osteoblastic cell proliferation, and differentiation during postnatal osteogenesis in human calvaria. *J. Cellular Biochem.* **64** 128-139

Dexter, T.M., Lajtha, L.G. (1974) Proliferation of haemopoietic stem cell *in vitro*. *Br. J. Haematol.* **28** 525-530

Dexter, T.M., Allen, T.M., Lajtha, L.G. (1977) Conditions controlling the proliferation of hemopoietic stem cells in vitro. *Cell. Phyiol.* **91** 335-349

Dexter, T.M. (1982) Stromal cell associated haemopoiesis. *J. Cell Physiol.* **1** 87-94

Dietrich, J.W., Canalis, E.M., Maina, D.M., Raisz. L.G. (1979) Effects of glucocorticoids on fetal rat bone collagen synthesis *in vitro*. *Endo.* **104** 715-721

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M., Campisi, J. (1995) A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc. Natl. Acad. Sci. USA* **92** 9363-9367

Dobnig, H., Turner, R.T. (1995) Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells. *Endo.* **136** 3632-3638

Ducy, P., Desbois, C., Boyce, B., Pinero, G., Story, B., Dunstan, C., Smith, E., Bonadio, J., Goldstein, S., Gundberg, C., Bradley, A., Karsenty, G. (1996) Increased bone formation in osteocalcin-deficient mice. *Nature* **382** 448-452

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A.L., Karsenty, G. (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* **89** 747-754

Duda, R.J.Jr., O'Brien, J.F., Katzmann, J.A. (1988) Concurrent assays of circulating bone gla-protein and bone alkaline phosphatase: effects of sex, age, and metabolic bone disease. *J. Clin. Endo. Metab.* **66** 951-957

Ebeling, P.R., Jones, J.D., Janes, C.H., Lane, A.W., O'Fallon, W.M., Riggs, B.L. (1992) Short-term effects of recombinant human insulin-like growth factor-I on bone turnover in normal women. *J. Bone Min. Res.* **7 (Suppl 1)** S138

Egrise, D., Martin, D., Vienne, A., Neve, P., Schoutens, A. (1992) The number of fibroblastic colonies formed from bone marrow is decreased and the *in vitro* proliferation rate of trabecular bone cells increased in aged rats. *Bone* **13** 355-361

Eriksen, E.F., Colvard, D.S., Berg, N.J., Graham, M.L., Mann, K.G., Spelsburg, T.C., Riggs, B.L. (1988) Evidence of estrogen receptors in normal human osteoblast-like cells. *Science* **241** 84-86

Fedarko, N.S., Vetter, U., Weinstein, S., Robey, P.G. (1992) Age-related changes in the hyaluronan, proteoglycan, collagen and osteonectin synthesis by human bone cells. *J. Cell. Phys.* **151** 215-227

Fei, R.G., Penn, P.E., Wolf, N.S. (1990) A method to establish pre-fibroblast and endothelial cell colony cultures from murine bone marrow. *Exp. Hematol.* **18** 953-957

Filshie, R.J.A. Zannettino, A.C.W., Marrynikola, V., Gronthos, S., Henniker, A.J., Bendall, L.J., Gottlib, D.J. (1998) MUC18, a member of the immunoglobulin superfamily, is expressed on bone marrow fibroblasts and a subset of hematological malignancies. *Leukemia* **12** 414-421

Filvarov, E.H., Derynck, R. (1996) Induction of myogenesis in mesenchymal cells by MyoD depends on their degree of differentiation. *Dev. Biol.* **178** 459-471

Flanagan, A.M., Chambers, T.J. (1992) Stimulation of bone nodule formation *in vitro* by prostaglandins E_1 and E_2. *Endo.* **130** 443-448

Franceschi, R.T. (1992) The role of ascorbic acid in mesenchymal differentiation. *Nutrition Rev.* **3** 65-67

Frenkel, B., Capparelli, C., Van Auken, M., Baran, D., Bryan, J., Stein, J.L., Stein, G.S., Lian, J.B. (1997) Activity of the osteocalcin promoter in skeletal sites of transgenic mice and during osteoblast differentiation in bone marrow-derived stromal cell cultures: effects of age and sex. *Endo.* **138** 2109-2116

Friedenstein, A.J., Chailakhjan, R.K., Lalikina, K,S. (1970) The development of fibroblast colonies in monolayer cultures of guinea pig marrow and spleen cells. *Cell Tiss. Kinet.* **3** 393-403

Friedenstein, A.J., Gorskaja, J.F., Kulagina, N.N. (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp. Hematol.* **4** 267-274

Friedenstein, A.J. (1980) Stromal mechanisms of bone marrow: cloning *in vitro* and transplantation *in vivo*. In: Immunology of bone marrow transplantation. ed. Thienfelder, S.: Springer-Verlag, Berlin 19-29

Friedenstein, A.J., Chailalhyan, R.K., Gerasimov, U.V. (1987) Bone marrow osteogenic stem cells: *in vitro* cultivation and transplantation in diffusion chambers. *Cell. Tiss. Kinet.* **20** 263-272

Friedenstein, A.J. (1990) Osteogenic stem cells in the bone marrow. *J. Bone Min. Res.* **7** 243-272

Friedenstein, A.J., Latzinik, N.V., Gorskaya, Y. F., Luria, E.A., Moskvina, I.L. (1992) Bone marrow stromal colony formation requires stimulation by haemopoietic cells. *Bone and Miner . Res.* **7**. eds Heersche, J.N.M., Kanis, J. Elsevier 199-213

Fuller, K., Gallagher, A.C., Chambers, T.J. (1992) Osteoblast resorption: stimulating activity is associated with the osteoblast cell surface and or the extracellular matrix. *Biochem. Biophys. Res. Comm.* **181** 67-73

Gallagher, J.A., Gundle, R., Beresford, J.N. (1996) Isolation and culture of bone forming cells (osteoblasts) from human bone. In: Methods in Molecular Medicine: Human cell Culture Protocols. eds. Jones, G.E., Totowa, N.J.: Humana Press 233-262

Gartner, S., Kaplan, H.S. (1980) Long-term culture of human bone marrow cells. *Proc. Natl. Acad. Sci. USA* **77** 4756-4759

Geoffroy, V., Ducy, P., Karsenty, G. (1995) A PEB2-alpha/AML-1-related factor increases osteocalcin promoter activity through its binding to an osteoblast-specific cis-acting element. *J. Biol. Chem.* **270** 30973-30979

Gilardetti, R.S., Chaibi, M.S., Stroumza, J., Williams, S.R., Antoniades, H.N., Carnes, D.C., Graves, D.T. (1991) High-affinity binding of PDGF-AA and PDGF-BB to normal human osteoblastic cells and modulation by interleukin-1. *Am. J. Physiol.* **261** C980-C985

Glowaki, J. (1995) Influence of age on human marrow. *Calcif. Tiss. Int.* **56 (Suppl 1)** S50-S51

Graham FL., Van der Eb, A.J. (1973) A new method for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52** 456-467

Graves, D.T., Valentin-Opran, A., Delgado, R., Valente, A.J., Mundy, G., Piche, J., (1989) The potential role of platelet-derived growth factor as an autocrine or paracrine factor for human bone cells. *Connect. Tiss. Res.* **23** 209-218

Grigoriadis, A.E., Heersche, J.N.M., Aubin, J.E. (1988) Differentiation of muscle, fat, cartilage and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. *J. Cell Biol.* **106** 2139-2151

Gronthos, S., Graves, S.E., Ohta, S., Simmons, P.J. (1994) The STRO-1^+ fraction of adult human bone-marrow contains the osteogenic precursors. *Blood* **84** 4164-4173

Gronthos, S., Simmons, P.J. (1995) The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions *in vitro*. *Blood* **85** 929-940

Gundle, R., Beresford, J.N. (1995) The isolation and culture of cells from explants of human trabecular bone. *Calcif. Tiss. Int.* **56** S8-S10

Gundle, R., Joyner, C.J., Triffitt, J.T. (1995) Human bone tissue formation in diffusion chamber culture *in vivo* by bone-derived cells and marrow stromal fibroblastic cells. *Bone* **16** 597-601

Hakeda, Y., Kawaguchi, H., Hurley, M., Pilbeam, C.C., Abreu, C., Linkhart, T.A., Mohan, S., Kumagawa, M., Raisz, L.G. (1996) Intact insulin-like growth factor binding protein-5 (IGFBP-5) associates with bone matrix and the soluble fragments of IGFBP-5 accumulated in culture medium of neonatal mouse calvariae by parathyroid hormone and prostglandin E_2-treatment. *J. Cell. Phys.* **166** 370-379

Hall, P.A., Watt, F.M. (1989) Stem cells: the generation and mainatenance of cellular diversity. *Development* **106** 619-633

Han, Z.H., Palnikar, S., Rao, D.S., Nelson, D., Parfitt, A.M. (1997) Effects of ethnicity and age or menopause on the remodelling and turnover of iliac bone implications for mechanisms of bone loss. *J. Bone Min. Res.* **12** 498-508

Harada, S., Matsumoto, T., Ogata, E.(1991) Role of ascorbic acid in the regulation of proliferation in osteoblast-like MC3T3-E1 cells. *J. Bone Min. Res.* **6** 903-908

Harris, H. (1990) The human alkaline phosphatases:what we know and what we don't know. *Clin. Chim. Acta* **186** 133-150

Harris, S.A., Enger, R.J., Riggs, B.L., Spelsburg, T.C. (1995) Development and characterization of a conditionally immortalised human fetal osteoblastic cell line. *J. Bone Min. Res.* **10** 178-186

Hata. R.I., Senoo, H.L. (1989) Ascorbic acid 2-phosphate stimulate collagen accumulation, cell proliferation, and formation of a three dimensional tissuelike substance by skin fibroblasts. *J. Cell. Phys.* **138** 8-16

Hauschka, P.V., Mavrakos, A.E., Iafrati, M.D., Doleman, S.E., Klagsbrun, M. (1986) Growth factors in bone matrix: isolation of multiple types by affinity chromatography on heparin-sepharose. *J. Biol. Chem.* **261** 12665-12674

Hauschka, P.V., Chen, T.L. Mavrakos, A.E. (1988) Polypeptide growth factors in bone matrix. *Ciba Foundation Symposium* **136** 207-225

Hayflick, L. (1965) The limited *in vitro* lifetime of human diploid cell strains. *Exp. Cell Res.* **37** 614-636

Haynesworth, S.E., Goshima, J., Goldberg, V.M., Caplan, A.I. (1992) Characterisation of cells with osteogenic potential from human marrow. *Bone* **13** 81-88

Heath, D. (1995) In "An introduction to experimental design and statistics for biology". UCL Press Limited. 275-272

Heath, D. (1995a) In "An introduction to experimental design and statistics for biology". UCL Press Limited. 222-231

Heldin, C.H., Ostman, A., Eriksson, A., Siebahn, A., Claesson-Welsh, L., Westermark, B. (1992) Platelet-derived growth factor: isoform-specific signalling via heterodimeric or homodimeric receptor complexes. *Kidney Int.* **41** 571-574

Herbertson, A., Aubin, J.E. (1992) Culture conditions promoting bone formation in rat marrow stroma also stimulate the growth and differentiation of other marrow lineages. *J. Bone Min. Res.* **7** S220

Herbertson, A., Aubin, J.E. (1995) Dexamethasone alters the subpopulation make up of rat bone marrow stromal cell cultures. *J. Bone Min. Res.* **10** 285-294

Herbertson, A., Aubin, J.E. (1995a) The effects of PDGF on rat bone marrow stromal populations. *J. Bone Min. Res.* **10** S410

Hicok, K.C., Thomas, T., Gori, F., Rickard, D.J., Spelsberg, T.C., Riggs, B.L. (1998) Development and characterization of conditionally immortalized osteoblast precursor cell lines from human bone marrow stroma. *J. Bone Min. Res.* **13** 205-217

Hirata, J., Keneko, S., Mishimura, J., Motomura, S., Ibayashi, H. (1985) Effect of PDGF and bone marrow-conditioned medium on the proliferation of human bone marrow-derived fibroblastoid colony-forming cells. *Acta. Hematol.* **71** 189-194

Horner, A., Bord, S., Kemp, P., Grainger, D., Compston, J.E. (1996) Distribution of platelet-derived growth factor (PDGF) A chain mRNA, protein, and PDGF-alpha receptor in rapidly forming human bone. *Bone* **19** 353-362

Houghton, A., Oyajobi, B.O., Foster, G.A., Russell, R.G.G., Stringer, B.M.J. (1998) Immortalization of human marrow stromal cells by retroviral transduction with a temperature sensitive oncogene: Identification of bipotential precursor cells capable of directed differentiation to either an osteoblast or adipocyte phenotype. *Bone* **22** 7-16

Hoyland, J.A., Mee, A.P., Baird, P., Braidman, I.P., Mawer, E.B., Freemont, A.J. (1997) Demonstration of eostrogen recptor mRNA in bone using *in situ* reverse-transcriptase polymerase chain reaction. *Bone* **20** 87-90

Hsieh, S.C., Graves,D.T. (1998) Pulse application of platelet-derived growth factor enhances formation of a mineralising matrix while continuous application is inhibitory. *J. Cellular Biochem.* **69** 169-180

Huang, J.S., Huang, S.S., Deuel, T.F. (1984) Specific covalent binding of platelet-derived growth factor to human plasma alpha 2 macroglobulin. *Proc. Natl. Acad. Sci.* USA **81** 342-346

Hughes, D.E., Boyce, B.F. (1997) Apoptosis in bone physiology and disease. *J. Clin. Pathology* **50** 132-137.

Hughes, F.J., McCulloch, C.A.G. (1991) Stimulation of the differentiation of osteogenic rat bone marrow stromal cells by osteoblast cultures. *Lab. Invest.* **64** 617-622

Hughes-Fulford, M., Appel, R., Kumegawa, M., Schmidt, J. (1992) Effect of dexamethasone on proliferation osteoblasts: inhibition of prostaglandin E_2 synthesis, DNA synthesis and alterations in actin cytoskeleton. *Exp. Cell Res.* **203** 150-156

Hurley, R.W., McCarthy, J.B., Verfaillie, C.M. (1995) Direct adhesion to bone marrow stroma via fibronectin receptors inhibits hematopoietic progenitor proliferation. *J. Clin. Invest.* **96** 511-519

Ibrahimi, A., Teboul, L., Gaillard, D., Amri, E., Ailhaud, G., Young, P., Cawthawn, M.A., Grimaldi, P.A. (1994) Evidence for a common mechanism of action for fatty acids and thiazolinedione antidiabetic agents on gene expression in preadipose cells. *Mol. Pharmacol.* **46** 1070-1076

Jee, W.S., Uneo, K., Deng, Y., Woodbury, S. (1985) The effect of prostaglandin E_2 in rapidly growing rats: increased metaphyseal hard tissue and cortico-endosteal bone formation. *Calcif. Tiss. Int.* **37** 148-157

Jee, W.S., Kimmel, D.B., Woodbury, S., Price, P., Woodbury, L.A. (1987) The role of bone cells in increasing metaphyseal hard tissue in rapidly growing rats treated with prostaglandin E_2. *Bone* **8** 171-178

Jilka, R.L., Weinstein, R.S., Takahashi, K., Parfitt, A.M., Manolagas, S.C. (1996) Linkage of decreased bone mass with impaired osteoblastogenesis in a murine model of accelerated senescence. *J. Clin. Invest.* **97** 1732-1740

Jilka, R.L. (1998) Cytokines, bone remodelling and estrogen deficiency: a 1998 update. *Bone* **23** 75-81

Jilka, RL, Weinstein RS, Bellido, T., Parfitt, A.M., Manolagas, S.C. (1998) Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. *J. Bone Min. Res.* **13** 793-802

Joyner, C.J., Bennet, A., Triffit, J.T. (1997) Identification and enrichment of human osteprogenitor cells by using differentiation stage-specific monoclonal antibodies. *Bone* **21** 1-6

Kappel, C.C., Velez-Yanguas, M.C., Hirschfeld, S., Helman, L.J. (1994) Human osteosarcoma cell lines are dependent on insulin-like growth factor I for *in vitro* growth. *Cancer Res.* **54** 2803-2807

Kasai, R., Bianco, P., Robey, P.G., Kahn A.J. (1994) Production and characterisation of antibody against the human bone GLA protein (BGP/osteocalcin) propeptide and its use in immunocytochemistry of bone cells. *Bone and mineral* **25** 167-182.

Kasperk, C., Wergedal, J., Strong, D., Farley, J., Wnagerin, K., Gropp, H., Ziegler, R., Baylink, D.J. (1995) Human bone cell phenotypes differ depending on their skeletal site of origin. *J. Clin. Endo. Metab.* **80** 2511-2517

Kassem, M., Ankersen, L., Eriksen, E.F., Clark, B.F.C., Rattan, S.I.S. (1997) Demonstration of cellular aging and senescence in serially passaged long-term cultures of human trabecular osteoblasts. *Osteoporosis Int.* **7** 514-524

Kasugai, S., Todescan, R., Nagata, T., Yao, K.L., Butler, W., Sodek, J. (1991) Expression of bone matrix proteins associated with mineralised tissue formation by adult rat bone marrow cells *in vitro*: inductive effects of dexamethasone on the osteoblastic phenotype. *J. Cell. Phys.* **147** 111-120

Kawaguchi, H., Pilbeam, C.C., Harrison, J.R., Raisz, L.G. (1995) The role of prostaglandins in the regulation of bone metabolism. *Clin. Orthop. Rel. Res.* **313** 36-46

Kelly, J.D., Raines, E.W., Ross, R., Murray, M.J. (1985) The B chain of PDGF alone is sufficient for mitogenesis. *EMBO J.* **4** 3399-3405

Kerby, J.A., Hattersley, G., Collins, D.A., Chambers, T.J. (1992) Derivation of osteoclasts from hematopoietic colony formation cells in culture. *J. Bone Min. Res.* **7** 353-362

Kiefer, F., Erwin, F.W., Keller, G. (1991) Fractionation of mouse bone marrow by adherence separates primitive hematopoietic stem cells from *in vitro* colony-forming cells and spleen colony-forming cells. *Blood* **78** 2577-2582

Klein-Nulend, J., Pilbeam, C.C., Harrison, J.R., Fall, P.M., Raize, L.G. (1991) Mechanism of regulation of prostaglandin production by parathyroid hormone, interleukin-1, and cortisol in cultured mouse parietal bones. *Endo.* **128** 2503-2510

Kohler, N., Lipton, A. (1974) Platelets as a source of fibroblast growth-promoting activity *Exp. Cell Res.* **87** 287-301

Komori, T., Yagi, A., Nomura, S., Yamaguchi, A., Saski, K., Deguchi, K., Skimizi, Y., Bronson, R.T., Gao, Y-H., Inada, M., Sato, M., Okamoto, R., Kitmura, Y., Yoshiki, S., Tishimoto, T. (1997) Targeted disruption of *cbfa1* results in a complete lack of bone formation owing to maturation arrest of osteoblasts. *Cell* **89** 755-764

Krebsbach, P.H., Kuznetsov, S.A., Satomura, K. Emmons, R.V.B., Rowe, D.W., Robey, P.G. (1997) Bone formation *in vivo*: comparison of osteogenesis by transplanted mouse and human marrow stromal fibroblasts. *Transplantation* **63** 1059-1069

Kuznetsov, S.A., Friedenstein, A.J., Robey, P.G. (1997) Factors required for bone marrow stromal fibroblast colony formation *in vitro*. *British J. Haematology* **97** 561-570

Kuznetsov, S.A., Krebsbach, P.H., Satomura, K., Kerr, J., Riminucci, M., Beneyahu, D., Robey, P.G. (1997a) Single colony-derived strained of human marrow stromal fibroblasts form bone after transplantation *in vivo*. *J. Bone Min. Res.* **12** 1335-1347

Kuznetsov, S.A., Robey, P.G. (1997) Species differences in growth requirements for bone marrow stromal fibroblast colony formation *in vitro*. *Calcif. Tiss. Int.* **59** 265-270

Laemmli, U.K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227** 680-685

Lanson, G.M., Katzmann, J.A., Kinlinger, T.K., O'Brien, J.F. (1985) Isolation and preliminary characterisation of a monoclonal antibody that interacts preferentially with the liver isoenzyme of human alkaline phosphatase. *Clin. Chem.* **31** 381-385

Leaffer, D., Sweeney, M., Kellerman, L.A., Avnur, Z., Krstenansky, J.L., Vickery, B.H., Caulfield, J.P. (1995) Modulation of osteogenic cell ultrastructure by RS-23581, an analogue of human parathyroid hormone (PTH)-related peptide (1-34) and bovine PTH (1-34). *Endo.* **136** 3624-3631

Leboy, P.S., Beresford, J.N., Devlin, C.D., Owen, M.E. (1991) Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. *J. Cell. Phys.* **146** 370-378

Leboy, P.S., Vaias, L., Uschmann, B., Golub, E., Adams, S.L., Pacifici, M. (1989) Ascorbic acid induces alkaline phosphatase, type X collagen, and calcium deposition in cultured chick chondrocytes. *J. Biol. Chem.* **264** 17281-17286

Lennon, D.P., Haynesworth, S.E., Bruder, S.P., Jaiwal, N., Caplan, A.I. (1996) Human and animal mesenchymal progenitor cells from bone marrow: identification of serum for optimal selection and proliferation. *In vitro Cell. Dev. Biol.* **32** 602-611

Lerner, U.F. (1996) Transforming growth factor-β stimulates bone resorption in neonatal mouse calvariae by a prostaglandin-unrelated but cell proliferation-dependent pathway. *J. Bone Min. Res.* **11** 1628-1639

Levine, M. (1986) New concepts in the biology and biochemistry of ascorbic acid. *New Engl. J. Med.* **314** 892-902

Lewin, B. (1994) Genes V: Oxford University Press: 1090-1095

Lian, J.B., Shalhoub, V., Aslam, F., Frenkel, B., Green, J., Hamrah, M., Stein, G.S., Stein, J.L. (1997) Species-specific glucocorticoid and 1,25-dihydroxyvitamin D responsiveness in mouse MC3T3-E1 osteoblasts: dexamethasone inhibits osteoblast differentiation and vitamin D down-regulates osteocalcin gene expression. *Endo.* **138** 2117-2127

Liang, C.T., Barnes, J., Seedor, J.G., Quartuccio, H.A., Bolander, M., Jeffrey, J.J., Fodan, G.A. (1992) Impaired bone activity in aged rats: alterations at the cellular and molecular level. *Bone* **13** 435-441

Lin, C.Q., Bissell, M.J. (1993) Multi-faceted regulation of cell differentiation by extracellular matrix. *FASEB J.* **7** 737-743

Lindsay, R. (1993) Prevention of osteoporosis. In: Primer on the metabolic bone diseases and disorders of bone and mineral metabolism. Raven Press, New York: 240-245

Locklin, R.M., Williamson, M.C., Beresford, J.N., Triffit, J.T., Owen, M.E. (1995) *In vitro* effects of growth factors and dexamethasone on rat marrow stromal cells. *Clin. Orthop. Rel. Res.* **313** 27-35

Long, M.W., Williams, J.L., Mann, K.G. (1990) Expression of human bone-related proteins in the hematopoietic microenvironment. *J. Clin. Invest.* **86** 1387-1395

Long, M.W., Robinson, J.A., Ashcraft, E.A., Mann, K.G. (1995) Regulation of human bone marrow-derived osteoprogenitor cells by osteogenic growth factors. *J. Clin Invest.* **95** 881-887

Lowik, C.W.G.M., Albias, M.J., van de Ruit, M., Papapoulos, S.E., van der Pluijm, G. (1993) Quantification of adherent and nonadherent cells cultured in 96-well plates using the supravital stain neutral red. *Analytical Biochem.* **213** 426-433

MacGregor, G.R., Nolan, G.P., Fiering, S., Roederer, M., Herzenberg, L.A. (1991) Use of E.coli lac Z (β–galactosidase) as reporter gene. *Meth. Mol. Biol.***7** 217-235

Majors, A.K., Boehm, C.A., Nitto, H., Midura, R.J., Muschler, G.F. (1997) Characterization of human bone marrow stromal cells with respect to osteoblastic differentiation. *J. Orthopaedic Res.* **15** 546-557

Maniatopoulos, C, Sodek, J., Melcher, A.H. (1988) Bone formation *in vitro* by stromal cells obtained from bone marrow of young adult rats. *Cell Tiss. Res.* **254** 317-330

Manolagas, S.C., Jilka R.L. (1995) Bone marrow, cytokines and bone remodelling: emerging insights into the pathophysiology of osteoporosis *N. Eng. J. Med.* **332** 305-311

Mardon, H.J., Bee, J. von der Mark, K., Owen, M.E. (1987) Development of osteogenic tissue in diffusion chambers from early precursor cells in bone marrow of adult rats. *Cell. Tiss. Res.* **250** 157-165

Marie, P.J., De Vernejoul, M.C. (1993) Proliferation of bone surface-derived osteoblastic cells and control of bone formation. *Bone* **14** 463-468

Marie, P.J., De Vernejoul, M.C., Lomri, A. (1992) Stimulation of bone formation in osteoporosis patients treated with fluoride associated with increased DNA synthesis by osteoblastic cells *in vitro*. *J. Bone Min. Res.* **7** 103-113

Marini J.C., Gerber, N.L. (1997) Osteogenesis imperfecta: rehabilitation and prospects for gene therapy. *JAMA* **277** 746-750

Mark, M.P., Prince, C.W., Gay, S. (1987) A comparative immunochemical study on the subcellular distributions of a 44 kDa bone phosphoprotein and bone gamma-carboxyglutamic acid (gla)-containing protein in osteoblasts. *J. Bone Min. Res.* **2** 337-346

Markose, E.R. Stein, J.L., Stein, G.S., Lian. J.B. (1990) Vitamin D-mediated modifications in protein-DNA interactions at two promoter elements of the osteocalcin gene. *Proc. Natl. Acad. Sci. USA* **87** 1701-1705

Marks, S.C., Hermey, D.C. (1996) The structure and development of bone. In: Principles of bone biology. eds. Bilezikian, J.P., Raisz, L.G., Rodan, G.A. Academic Press. 3-14

Martin, R.B., Zissimos, S.L. (1991) Relationships between marrow fat and bone turnover in ovariectomized and intact rats. *Bone* **12** 123-131

McCarthy, T.L., Centrella, M., Canalis, E. (1989) Regulatory effects of insulin-like growth factors I and II on bone collagen synthesis in rat calvarial cultures. *Endo.* **124** 301-309

McCarthy, T.L., Centrella, M., Canalis, E. (1990) Cyclic AMP induces insulin-like growth factor I synthesis in osteoblast-enriched cultures. *J. Biol. Chem.* **265** 15353-15356

McCarthy, T.L., Centrella, M., Raisz, L.G., Canalis, E. (1991) Prostaglandin E_2 stimulates insulin-like growth factor I synthesis in osteoblast-enriched cultures from fetal rat bone. *Endo.* **128** 2895-2900

McCauley, L.K., Koh, A.J., Beecher, C.A., Cui, Y.Q., Decker, J.D., Francheschi, R.T., (1995) Effects of differentiation and transforming growth-factor beta 1 on PTH/PTHRp receptor messenger-RNA levels in MC3T3-E1 cells. *J. Bone Min. Res.* **10** 1243-1255

McCauley, L.K., Koh, A.J., Beecher, C.A., Cui, Y.Q., Rosol, T.J., Francheschi, R.T., (1996) PTH/PTHRp receptor is temporally regulated during osteoblast differentiation and is associated with collagen-synthesis. *J. Cell. Biochem.* **61** 638-647

McIntyre, A.P., Bjornson, B.H. (1986) Human bone marrow stromal cell colonies: response to hydrocortisone and dependence on platelet-derived growth factor. *Exp. Hematol.* **14** 833-839

Meunier, P.J., Aaron. J., Edouard, C.,Vignon, G. (1971) Osteoporosis and the replacement of cell populations of the marrow by adipose tissue. *Clin. Orthop. Rel. Res.* **80** 147-154

Mitlak, B.H., Finkelman, R.D., Hill, E.L., Li, J., Martin, B., Smith, T., D'Andrea, M., Antoniades, H.N., Lynch, S.E. (1996) The effect of systemically administered PDGF-BB on the rodent skeleton. *J. Bone Min. Res.* **11** 238-247

Miyauchi, A., Alvarez, J., Greenfield, E.M., Teti, A., Grano, M., Colucci, S., Zambonin-Zallone, A., Ross, F.P., Teitelbaum, S.L., Cheresh, D., Hruska, K.A. (1991) Recognition of osteopontin and related peptides by an alpha v beta 3 integrin stimulates immediate cell signals in osteoclasts *J. Biol. Chem.* **266** 20369-20374

Mochizuki, H., Hakeda, Y., Wakatsuki, N., Usui, N., Akashi,S., Sato, T., Tanaka,K., Kumegawa, M. (1992) Insulin-like growth factor-1 supports formation and activation of osteoclasts. *Endo.* **131** 1075-1080

Mohan, S. (1993) Insulin-like growth factor binding proteins in bone cell reulation. *Growth Regulation.* **3** 67-70

Mori, S., Jee, X. Li, J. (1992) Production of new trabecular bone in osteopaenic ovariectomised rats by prostaglandin E_2. *Calcif. Tiss. Int.* **50** 80-87

Morike, M., Schultz, M., Brenner, R.E., Bushart, G.B., Teller, W.M., Vetter, U. (1993) *In vitro* expression of osteoblastic markers in cells isolated from normal fetal and postnatal human bone and from bone of patients with osteogenesis imperfecta. *J. Cell. Phys.* **157** 439-444

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65** 55-63

Mosselman, S., Polman, J., Dijkema, R. (1996) ERβ: identification and characterization of a novel human estrogen receptor. *FEBS Letters* **392** 49-53

Muschler, G.F., Boehm, C., Easley, K. (1997) Aspiration to obtain osteoblast progenitor cells from human bone marrow: the influence of aspiration volume. *J. Bone Joint Surg. Am.* **79A** 1699-1709

Nakahara, H., Bruder, S.P., Haynesworth, S.E., Holecek, J.J., Baber, M.A., Goldberg, V.M., Caplan, A.I. (1990) Bone and cartilage formation in diffusion chambers by subcultured cells derived from the periosteum. *Bone* **11** 181-188

Nash, T.J., Howlett, C.R., Martin, C., Steel, J., Johnson, K.A., Hicklin, D.J. (1994) Effect of platelet-derived growth factor in tibial osteotomies in rabbits. *Bone* **15** 203-208

Neave, H.R. (1981) In "Elementary statistics tables". Routledge: 28-34

Nesbitt, S.A. Horton, M.A. (1997) Trafficking of matrix collagens through bone-resorbing osteoclasts. *Science* **276** 266-269

Nicolas, V., Mohan, S., Honda, Y., Prewett, A., Finkelman, R.D., Baylink, D.J., Farley, J.R. (1995) An age-related decrease in the concentration of insulin-like growth factor binding protein-5 in human cortical bone. *Calcif. Tiss. Int.* **57** 206-212

Nicolas, V., Prewett, A., Bettica, P., Mohan, S., Finkelman, R.D., Baylink, D.J., Farley, J.R. (1994) Age-related decreases in insulin-like growth factor-I and transforming growth factor beta in femoral cortical bone from both men and women: implications for bone loss with aging. *J. Clin. Endo. Metab.* **78** 1011-1016

Nimni, M.E., Bernick, S., Ertl, D., Nishimoto, S.K., Paule, W., Strates, B.S. Villanueva, J. (1988) Ectopic bone formation is enhanced in senescent animals implanted with embryonic cells. *Clin. Orthop. Rel. Res.* **234** 255-266

Nishida, A., Yamaguchi, A., Tanizawa, T., Endo, N., Mashiba, T., Uchiyama, Y., Suda, T., Yoshiki, S., Takahashi, H.E. (1994) Increased bone formation by intermittent parathyroid hormone administration is due to the stimulation of proliferation and differentiation of osteoprogenitor cells in bone marrow. *Bone* **15** 717-723

Nomura, H., Ishiguro, T., Morimoto, S. (1969) Studies on L-ascorbic acid derivatives. III. Bis(L-ascorbic acid-3,3') phosphate and L-ascorbic acid 2-phosphate. *Chem. Pharm. Bull.* **17** 387-393

Norrdin, R.W., Shih, M.S. (1988) Systemic effects of prostaglandin E_2 on vertebral trabecular remodelling in beagles used in healing study. *Calcif. Tis. Int.* **42** 323-368

Nuttall, M.E., Patton, A.J. Olivera, D.L., Nadeau, D.P., Gowen, M. (1998) Human trabecular bone cells are able to express both osteoblastic and adipocytic phenotype: implications for osteopenic disorders. *J. Bone Min. Res.* **13** 371-382

Oates, T.W., Kose, K.N., Xie, J.F., Graves, D.T., Collins, J.M., Cochran, D.L. (1995) Receptor binding of PDGF-AA and PDGF-BB, and the modulation of PDGF receptors by TGF-β, in human periodontal ligament cells. *J. Cell. Phys.* **162** 359-366

Ogiso, B., Hughes, F.J., Melcher, A.H., McCulloch, C.A.G. (1991) Fibroblasts inhibit mineralised bone nodule formation by rat bone marrow stromal cells *in vitro*. *J. Cell. Phys.* **146** 442-450

Oldberg, A., Franzen, G.J., Heinegard, D. (1986) Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell binding sequence. *Proc. Natl. Acad. Sci. USA* **83** 8819-8823

Ono, M., Yasuaki, A., Kitagawa, I., Kitagawa, Y. (1990) Ascorbic acid phophate stimulates type IV collagen synthesis and accelerates adipose conversion of 3T3-L1 cells. *Exp. Cell Res.* **187** 309-914

Oreffo, R.O.C., Virdi AS, Triffit, J.T. (1995) Evidence for maintenance of non-adherent osteoprogenitors in cultures of human bone marrow. *J. Bone Min. Res.* **10** S208

Oreffo, R.O.C., Bennett A, Triffit, J.T. (1996) Osteoblast stem-cell number and human aging. *J. Bone Min. Res.* **11** S391

Oreffo, R.O.C., Bord, S., Triffit, J.T. (1998) Skeletal progenitor cells and ageing human populations. *Clin. Sci.* **94** 549-555

Otto, F., Thonell, A.,P., Crompton, T., Denzel. A., Gilmore., K.C., Rosewell., I.R., Stamp., G.W.H. Beddington, R.S.P., Mundlos, S., Olsen, B.R., Selp, P.B., Owen, M.J. (1997) cbfa1 a candidate gene for cleidocranial dysplasia syndrome is essential for osteoblast differentiation and bone development. *Cell* **89** 765-771

Owen, M.E., Cave, J., Joyner, C.J. (1987) Clonal analysis *in vitro* of osteogenic differentiation of marrow CFU-F. *J. Cell Sci.* **87** 731-738

Owen, M.E. (1988) Marrow stomal stem cells. *J. Cell Sci. Supp.* **10** 63-76

Owen, T.A., Aronow, M.S., Shalhoub. V., Barone, L.M., Wilming, L., Tassinari, M., Kennedy, M.B., Pockwinse, S., Lian, L.B., Stein, G.S., (1990) Progressive development of the rat osteoblast phenotype *in vitro* reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J. Cell. Physiol.* **143** 420-430

Patt, H.M., Maloney, M.A., Flannery, M.L. (1982) Hematopoietic microenvironment transfer by stromal fibroblasts derived from bone marrow varying in cellularity. *Exp. Hematol.* **10** 728-732

Pead, M.J., Lanyon, L.E. (1989) Indomethacin modulation of load-related stimulation of new bone formation *in vivo*. *Calcif. Tiss. Int.* **45** 34-40

Peden, K.W.C., Charles, C., Sanders, L., Tennekoon, G.I. (1989) Isolation of rat schwann cell lines: use of SV40 T antigen gene regulated by synthetic metallothionein promoters. *Exp. Cell Res.* **185** 60-72

Penn, P.E., Jiang, D.Z., Fei, R.G., Sitnicka, E., Wolf, N.S. (1993) Disecting the hematopoietic microenvironment. IX. Further characterization of murine bone marrow stromal cells. *Blood* **81** 1205-1213

Pereira, R.F., Halford, K.W., Ohara, M.D., Leeper, D.B., Sokolov, B.P., Pollard, M.D., Bagasra, O., Prockop, D.J. (1995) Cultured adherent cells from marrow can serve as long lasting precursor cells for bone, cartilage, and lung in irradiated mice. *Proc. Soc. Natl. Acad. Sci. USA* **92** 4857-4861

Peterkofsky, B. (1972) The effects of ascorbic on collagen polypeptide synthesis and proline hydroxylation during the growth of cultured fibroblasts. *Arch. Biochem. Biophys.* **152** 318-328

Pfeilschifter, J., Oechsner M., Naumann, A., Gronwald, R.G.K., Minne, H.W., Ziegler, R. (1990) Stimulation of bone matrix apposition *in vitro* by local growth factors: a comparison between insulin-like growth factor I, platelet-derived growth factor, and transforming growth factor β. *Endo.* **127** 69-75

Pfeilschifter, J., Bonewald, L., Mundy, G. (1990a) Characterisation of the latent transforming growth factor β complex in bone. *J. Bone Min. Res.* **5** 49-58

Pfeilschifter, J., Diel, I., Pilz, U., Brunotte, K., Naumann, A., Ziegler, R. (1993) Mitogenic responsiveness of human bone cells *in vitro* to hormones and growth-factors decreases with age. *J. Bone Min. Res.* **8** 707-717

Piche, J.E., Graves, D.T. (1989) Study of the growth factor requirements of human bone-derived cells: A comparison with human fibroblasts. *Bone* **10** 131-138

Pietrzkowski, Z. ,Wernicke, D., Porcu, P., Jameson, B.A., Baserga, R. (1992) Inhibition of cellular proliferation by peptide analogues of insulin-like growth factor 1. *Cancer Res.* **52** 6447-6451

Pilbeam, C.C., Raisz, L.G., Voznesensky, O., Alander, C.B., Delman, B.N., Kawaguchi, H. (1995) Autoregulation of inducible prostglandin G/H synthase in osteoblastic cells by prostaglandins. *J. Bone Min. Res.* **10** 406-414

Pockwinse, S.M., Wilming, L.G., Conlon, D.M., Stein, G.S., Lian, J.B. (1992) Expression of cell growth and bone specific genes at single cell resolution during development of bone tissue-like organisation in primary osteoblast cultures. *J. Cell. Biochem.* **49** 310-323

Price, PA., Parthemore JG., Deftos, LJ. (1980) New biochemical marker for bone metabolism. *J. Clin. Invest.* **66** 878-883

Price, T.N.C., Moorwood, K., James, M.R., Burke, J.F., Mayne, L.V. (1994) Cell cycle progression, morphology and contact inhibition are regulated by the amount of SV40 T antigen in immortal human cells. *Oncogene* **9** 2897-2904

Prokop, D.J. (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276** 71-74

Puzas, J.E. (1993) The osteoblast. In: Primer on the metabolic bone diseases and disorders of bone and mineral metabolism. Raven Press New York: 15-21

Quarto, R., Thomas, D., and Liang, C.T. (1995) Bone progenitor-cell deficits and the age-associated decline in bone repair capacity. *Calcif. Tiss. Int.* **56** 123-129

Rahman, S., Oberdorf, A.,Montecino, M., Tanhauser, S.M., Lian, J.B., Stein, G.S., Laipis, P.J., Stein, J.L. (1993) Multiple copies of the bone-specific osteocalcin gene in mouse and rat. *Endo.* **133** 3050-3053

Raisz, L.G. (1993) Bone cell biology: New approaches and unanswered questions. *J. Bone Min. Res.* **8** S457-S465

Raisz, L.G., Alander, C.B., Fall, P.M., Simmons, H.A. (1990) Effects of prostglandin $F_{2\alpha}$ on bone formation and resorption in cultures neonatal mouse calvariae: role of prostaglandin E_2 production. *Endo.* **126** 1654-1659

Raisz, L.G., Fall, P.M. (1990) Biphasic effects of prostaglandin E_2 on bone formation in cultured fetal rat calvariae: interaction with cortisol. *Endo.* **128** 1654-1659

Raisz, L.G., Fall, P.M., Gabbitas, B.Y., McCarthy, T.L., Kream, B.E., Canalis, E. (1993) Effects of prostglandin E_2 on bone formation in cultured fetal rat calvariae: role of insulin-like growth factor-I. *Endo.* **133** 1504-1510

Rang, H.P., Dale, M.M., Ritter, J.M. (1995) Pharmacology, Churchill livingstone, U.K. 230-234

Reed, B.Y., Zerwekh, J.E., Sakhaee, K., Breslau, N.A., Gottschalk, F., Pak, C.Y.C. (1995) Serum IGF 1 is low and correlated with osteoblastic surface in idiopathic osteoporosis. *J. Bone Min. Res.* **10** 1218-1224

Reid, D.M., Nicoll, J.J., Smith, M.A., Higgins, B., Tothill, P., Nuki, G. (1986) Corticosteroids and bone mass in asthma: comparisons with rheumatoid arthritis and polymyalgia rheumatica. *Br. Med. J.* **293** 1463-1466

Reid, I.R., Chapman, G.E., Fraser, T.R.C., Davies, A.D., Surus, A.S., Meyer, J. Hyq., N.L., Ibbertson, H.K. (1986a) Low serum osteocalcin levels in glucocorticoid-treated asthmatics. *J. Clin. Endo. Metab.* **62** 379-383

Remegen, W. (1989) Illustration in: Osteoporosis. Sandoz Ltd. p21

Rickard, D.J., Kassem, M., Hefferan, T.E., Sarkar, G., Spelsberg, T.C., Riggs, B.L. (1996) Isolation and characterisation of osteoblast precursor cells from human bone marrow. *J. Bone Min. Res.* **11** 312-324

Rickard, D.J., Kazhdan, I., Leboy, P.S. (1995) Importance of 1,25-dihydroxyvitamin D_3 and the non-adherent cells of marrow for osteoblast differentiation from rat marrow stromal cells. *Bone* **16** 671-678

Rickard, D.J., Sullivan, T.A., Shenker, B.J., Leboy, P.S., Kazhdan, I. (1994) Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. *Dev. Biol.* **161** 218-228

Riggs, B.L., Melton, L.J. (1986) Medical progress series: Involutional osteoporosis. *New Engl. J. Med.* **314**: 1676-1686

Riggs, B.L., Khosla, S., Melton, L.J. (1998) A unitiary model for invoutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in post-menopausal women and contributes to bone loss in aging men. *J. Bone Min. Res.* **13** 763-773

Robey, P.G., Fedarko, N.S., Hefferan, T.E., Bianco, P., Vetter, U.K., Grzeski, W., Friedenstein, A., Van der Pluilm, G., Mintz, K.P., Young, M.F., Kerr, J.M., Ibaraki, K., Heegaard, A.M. (1993) Structure and molecular regulation of bone matrix proteins. *J. Bone Min. Res.* **8** S483-S487

Rodan, G. (1991) Autocrine/paracrine regulation of osteoblast growth and differentiation. *Lab. Invest.* **64** 593-595

Rodan, G.A., Harada, S.I., (1997) The missing bone. *Cell* **89** 677-680.

Rosen, C.J., Usiskin, K., Owens, M., Barlascini, C.O., Belsky, M., Adler, R.A. (1990) T lymphocyte surface antigen markers in osteoporosis. *J. Bone. Min. Res.* **5** 851-855

Ross, R., Glomset, J., Kariya, B., Harker, L. (1974) A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells *in vitro*. *Proc. Natl. Acad. Sci. USA.* **71** 1207-1210

Rouleau, M.F., Mitchell, J., Goltzman, D. (1988) *In vivo* distribution of parathyroid hormone receptors in bone: evidence that a predominant osseous target cell is not the mature osteoblast. *Endo.* **123** 187-191

Rouleau, M.F., Mitchell, J., Goltzman, D. (1990) Characterisation of the major parathyroid hormone target cell in the endosteal metaphysis of rat long bones. *J. Bone Min. Res.* **5** 103-1053.

Rozman, C., Feliu, E., Berga, L., Reverter, J.C., Climent, C., Ferran, M.J. (1989) Age-related variations of fat tissue fraction in normal bone marrow depend both on size and number of adipocytes: a stereological study. *Exp. Hematol.* **17** 43-37

Rungby, J., Kassem, M., Eriksen, E.F., Danscher, G. (1993) The von Kossa reaction for calcium deposits: silver lactate staining increases sensitivity and reduces background. *Histochem. J.* **25** 446-451

Rydziel, S., Ladd, C., McCarthy, T.L., Centrella, M., Canalis, E. (1992) Determination and expression of platelet-derived growth factor-AA in bone cell cultures. *Endo.* **130** 1916-1922

Sarma, U., Edwards, M., Motoyoshi, K., Flannagan, A.M. (1998) Inhibition of bone resorption by 17 beta-estradiol in human bone marrow cultures. *J. Cell. Phys.* **175** 99-108

Schubert, D. (1992) Collaberative interactions between growth factors and the extracellular matrix. *TICB* **2** 63-66

Scutt, A., Bertram, P. (1995) Bone marrow cells are targets for the anabolic actions of prostaglandin E_2 on bone: induction of a transition from nonadherent to adherent osteoblast precursors. *J. Bone Min. Res.* **10** 474- 487

Scutt, A., Zeschnigk, M., Bertram, P. (1995) PGE_2 induces the transition from non-adherent to adherent bone marrow mesenchymal presursor cells via a cAMP/EP_2-mediated mechanism. *Prostaglandins* **49** 383-395

Scutt, A., Bertram, P., Brautigam, M. (1996) The role of glucocorticoids and prostaglandin E_2 in the recruitment of bone-marrow mesenchymal cells to the osteoblastic lineage - positive and negative effects. *Calcif. Tiss. Int.* **59** 154-162

Shalhoub, V., Conlon, D., Tassinari, M., Quinn, C., Partridge, N., Stein, G.S., Lian, J.B. (1992) Glucocorticoids promote development of the osteoblast phenotype by selectively modulating expression of cell growth and differentiation associated genes. *J. Cell Biochem.* **50** 425-440

Shalhoub, V., Aslam, F., Breen, E., van Wijnen, A., Bortell, R., Stein, G.S., Stein, J.L., Lian, J.B. (1998) Multiple levels of steroid hormone dependent control of osteocalcin during osteoblast differentitaion: glucocorticoid regulation of basal and vitamin D stimulated gene expression. *J. Cell Biochem.* **69** 154-168

Sharpe, P.M., Ferguson. M.W.J. (1988) Mesenchyme influence on epithelial differentiation in developing systems. *J. Cell Sci. Suppl.* **10** 195-230

Shay, J.W., Wright, W.E. (1989) Quantitation of the frequency of immortalization of normal human diploid fibroblasts by SV40 large T-antigen. *Exp. Cell Res.* **184** 109-118

Shay, J.W., Werbin, H. (1991) Defining the molecular mecanisms of human cell immortalization. *Biochim. Biophys. Acta.* **1072** 1-7

Shigeno, Y. Ashton, B.A. (1995) Human bone-cell proliferation *in vitro* decreases with human donor age. *J. Bone Joint Surg.* **77b** 139-142

Shinar, D.M., Endo, N., Halperin, D., Rodan, G.,A., Weinreb, M. (1993) Differentitial expression of insulin like growth factor I, IGF-I and IGF-II messenger ribonucleic acid in growing rat bone. *Endo.* **132** 1158-1167

Simmons, D.J., Seitz, P., Kidder, L., Klein, G.L., Waeltz, M., Gundberg, C.M., Tabuchi, C., Yang, C., Zhang, R.W. (1991) Partial characterisation of rat marrow stromal cells. *Calcif. Tiss. Int.* **48** 326-334

Simmons, P.J., Przepiorka, D., Thomas, E.D., Torok-Storb, B. (1987) Host origin of marrow stromal cells following allogeneic bone marrow transplantation. *Nature* **328** 429-432

Simmons, P.J., Torok-Storb, B. (1991) Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood* **78** 55-62

Smith, W.L. (1992) Prostanoid biosynthesis and mechanisms of action. *Am. J. Physiol.* **263** F181-F191

Smithies, O., Gregg, R.G., Boggs, S.S., Koralewski, M.A., Kucherlapati, R.S. (1985) Insertion of DNA sequences into the human chromosomal β-globin locus by homologous recombination. *Nature* **317** 230-234

Song, Z.X., Quesenberry, P.J. (1984) Radioresistant murine marrow stromal cells: a morphologic and functional characterisation. *Exp. Hematol.* **12** 523-533

Song, Z.X., Shadduck, J.K., Innes, D.J., Waheed Jr, A., Quesenberry, P.J. (1985) Hematopoietic factor production by a cell line (TC-1) derived from adherent murine mouse marrow cells. *Blood* **66** 273-281

Southern, E. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98** 503-507

Spelsberg, T.C., Harris, S.A., Riggs, B.L. (1995) Immortalized osteoblast cell systems (new fetal osteoblast systems). *Calcif. Tiss. Int.* **56 (Suppl 1)** S18-S21

Stahl, H., Knippers, R. (1987) The simian virus 40 large tumour antigen. *Biochim. Biophys. Acta.* **910** 1-10

Stein, G.S., Lian, J.B., Owen, T.A. (1990) Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. *FASEB* **4** 3111-3123

Stepan, J.J., Silinkova-Malkova, E., Havrenek, T. (1983) Relationship of plasma tartrate-resistant acid phosphatase to the bone isoenzyme of serum alkaline phosphatase in hyperparathyroidism. *Clin. Chim. Acta.* **133** 189-200

Stewart, K., Screen, J., Jefferiss, C.M., Walsh, S., Beresford, J.N. (1996) Co-expression of the STRO-1 antigen and alkaline phosphatase in cultures of human bone and marrow cells *J. Bone Min. Res.* **11 (Suppl)** S142

Stewart, K., Jefferiss, C., Screen, J., Walsh, S., Beresford, J.N. (1998) The identification and isolation of cells of the osteoblast lineage in cultures of adult human bone-derived cells by dual labeling with the monoclonal antibodies STRO-1 and B4-78. In press: Ernst Schering Foundation: eds. Russel, R.G.G., Skerry, T., Kollenkirchen, U. 43-59

Suda, T., Udagawa, N., Takahashi, N. (1996) Cells of bone: Osteoclast generation. In: Principles of bone biology. eds. Bilezikian, J.P., Raisz, L.G., Rodan, G.A. Academic Press. 87-102

Takagaki, Y.M., Suzuki, Y., Kawase, T., Saito, S. (1996) Distinct responses of different populations of bone cells to mechanical stress. *Endo.* **137** 2029-2035

Tanaka, H., Liang, C.T. (1995) Effect of platelet-derived growth factor on DNA synthesis and gene expression in bone marrow stromal cells derived from adult and old rats. *J. Cell. Phys.* **164** 367-375

Tang, L.Y., Kimmel, D.B., Jee, W.S.S., Yee, J.A. (1996) Functional characterisation of prostaglandin E_2 inducible osteogenic colony forming units in culture of cell isolated from the neonatal rat calvarium. *J. Cell. Phys.* **166** 76-83

Tashjian, A.H., Voelkel, E.F., Lazzoro, M., Singer, F.R., Roberts, A.B., Derynck, R., Winkler, M.E., Levine, L. (1985) α and β transforming growth factors stimulate prostglandin production and bone resportion in cultured mouse calvaria. *Proc. Natl. Acad. Sci. USA.* **82** 4535-4538

Teboul, L., Gaillard, D., Staccini, L., Inadera, H., Amri, E., Grimaldi, P.A. (1995) Thiazolidinediones and fatty acids convert myogenic cells into adipose like cells. *J. Biol. Chem.* **270** 28183-28187

Tontonoz, P., Hu, E., Spiegelman, B.M. (1994) Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. *Cell* **79** 1147-1156

Tsuji, T., Hughes, F.J., McCulloch, C.A.G., Melcher, A.H. (1990) Effects of donor age on osteogenic cells of rat bone. *Mech. Ageing Dev.* **51**: 121-132

Turksen, K., Aubin J.E. (1991) Positive and negative immunoselection for enrichment of two classes of osteoprogenitor cells. *J. Cell. Biol.* **114** 373-384

Uchiyama, Y., Miyama, K., Kataginri, T., Yamaguchi, A., Takamori, H., Nkashima, K., Sato, T., Suda, T. (1994) Adipose conversion is accelerated in bone marrow cells of congenitally osteoporotic SAMP6 mice. *J. Bone Min. Res.* **9 (Suppl 1)** S321

Udegawa, N., Takahashi, N., Akatsu, T., Sasaki, T., Yamaguchi, A., Kodma, H., Martin, T.J., Suda, T. (1989) The bone marrow-derived stromal cell lines MC3T3-G2/PA6 and ST2 support osteoclast-like cell differentiation in cocultures with mouse spleen cells. *Endo.* **125** 1805-1813

Ueda, K., Saito, A., Nakano, H., Aoshima, M., Yokota, M., Muraoka, R., Iwaya, T. (1980) Cortical hyperstosis following long-term administration of prostaglandin E_1 in infants with cyanotic congenital heart disease. *J. Pediatr.* **97**, 834-836

van der Plas, A., Aarden, E.M., Feijen, J.H.M., Deboer, A.H., Wiltink, A., Alblas, M.J., de Leij, L., Nijweide, P.J. (1994) Characteristics and properties of osteocytes in culture. *J. Bone Min. Res.* **9** 1697-1704

Waller, E.K., Huang, S., Terstappen, L. (1995) Changes in the growth properties of $CD34^+$, $CD38^-$ bone marrow progenitors during human fetal development. *Blood* **86** 710-718

Walsh, S., Beresford, J.N. (1995) Differentiation of human marrow stromal cells: effects of dexamethasone, basic fibroblast growth factor and transforming growth factor beta. *Bone* **16** 690 P14

Wang, E., Wang, J., Chin, E., Zhou, J., Bondy, C.A. (1995) Cellular patterns of insulin-like growth factor system gene expression in murine chondrogenesis and osteogenesis. *Endo.* **136** 2741-2751

Weinreb, M., Shinar, D., Rodan, G.A. (1990) Different pattern of alkaline phosphatase, osteopontin and osteocalcin expression in developing rat bone visualised by *in situ* hybridisation. *J. Bone Min. Res.* **5** 831-842

Weinreb, M., Suponitzky, I., Keila, S. (1997) Systemic administration of an anabolic dose of PGE_2 in young rats increases the osteogenic capacity of bone marrow. *Bone* **20** 521-526

Wetterwald, A., Hoffstetter, W., Cecchini, M.G. *et al.* (1996) Characterisation and cloning of the E11 antigen, a marker expressed by rat osteoblasts and osteocytes. *Bone* **18** 125-132

Whitfield, J.F., Morely, P. (1995) Small bone building fragments of parathyroid hormone: new therapeutic agents for osteoporosis. *TIPS* **16** 382-387

Woodiel, F.N, Fall, P.M., Raisz, L.G. (1996) Anabolic effects of prostaglandins in cultured fetal rat calvariae: structure-activity relations and signal transduction pathway. *J. Bone Min. Res.* **11** 1249-1255

Wright, W.E., Perira-Smith, O.M., Shay, J.W. (1989) Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts. *Mol. Cell Biol.* **9** 3088-3092

Xiao, G.Z., Cui, Q., Ducy, P., Karsenty, G., Franceschi, R.T. (1997) Ascorbic acid-dependent activiation of the osteocalcin promoter in MC3T3-E1 preosteoblasts: requirement for collagen matrix synthesis and the presence of an intact OSE2 sequence. *Mol. Endo.* **11** 1103-1113

Yu, X., Hsieh, S., Bao, W., Grave, D.T. (1997) Temporal expression of PDGF receptors and PDGF regulatory effects on osteoblastic cells in mineralizing cultures. *Am. J. Physiol.* **272** C1709-1719

Zaidi, M., Alam, A.S.M.T., Shankar, V.S., Bax, B.E., Bax, C.M.R,. Moonga, B.S., Bevis, P.J.R., Stevens, C., Blake, D.R., Pazianas, M., Huang, C.L.H. (1993) Cellular biology of bone resorption. *Biological Reviews of the Cambridge Philosophical Society* **68** 197-264

Zhang, L., Leeman, E., Carnes, D.C., Graves, D.T. (1991) Human osteoblasts synthesize and respond to platelet-derived growth factor. *Am. J. Physiol.* **261** C348-C351

Zimmerman, B. (1992) Degeneration of osteoblasts involved in intramembranous ossification of fetal rat calvaria. *Cell. Tiss. Res.* **267** 75-84